

REMARKS

Applicants have carefully studied the Office Action mailed on November 18, 2005, which issued in connection with the above-identified application. The present amendments and remarks are intended to be fully responsive to all points of rejection raised by the Examiner and are believed to place the claims in condition for allowance. Favorable reconsideration and allowance of the present claims are respectfully requested.

I. October 27, 2005 Telephonic Interview With Examiner Prema Maria Mertz

Applicants gratefully acknowledge the courtesy shown by Examiner Prema Maria Mertz, during the informal telephonic interview with Applicants' representatives, Lisa D. Tyner and Chandra E. Garry, on Wednesday, November 2, 2005.

During the interview, the Applicants' representatives explained that the claim term "human" in claim 1 is supported in the specification based on disclosure of the specific term on p. 1, l. 13 of the specification and recitation of particular amino acid residues in Table 2 on p. 12 of the specification. Applicants' representatives further proposed submitting an alignment of the sequences of the β_c chain of cytokine receptor from various species demonstrating that, the amino acid residues disclosed in Table 2 on p. 12 correspond to the human β_c chain sequence. The Examiner agreed that such information would assist in determining whether one having ordinary skill in the art would have understood that the disclosed domain of D4 β_c is of human origin.

Thus, as discussed during the telephonic interview and in greater detail below, Applicants submit herewith, as **Exhibit A**, a copy of a sequence alignment comparing the sequence of the β_c chain of the human cytokine receptor with the sequences of mouse, rat, guinea pig, bovine and canine β_c chains. This sequence alignment demonstrates that the disclosed D4 β_c is of human origin.

II. Status Of The Claims

Claims 1, 2, 4-6, 8, 10-34 and 36-40 were pending in the present application. Claims 1, 2, 4-6, 18, 32-34, and 36-40 have been amended to correct for formal matters and in order to more

particularly point out and distinctly claim the invention. Support for the term “group consisting of GM-CSF receptor, IL-3 receptor, and IL-5 receptor” recited in claim 1 can be found, for example, in the specification on p. 3, ll. 29-30, p. 7, ll. 26-27, p. 8, ll. 13-14, and p. 14, ll. 3-4, and in claim 10 as originally filed. Support for the term “B'-C' loop comprising any one of the following residues Lys362, Met363, Arg364, Tyr365, Glu366, and His367, or a combination thereof” recited in claim 1 can be found, for example, in the specification on p. 10, l. 35, p. 11, ll. 1-6, p. 26, l. 18, p. 27, ll. 1-4, and p. 29, ll. 2-3. Support for the term “F'-G' loop comprising any one of the following residues Thr416, Arg418, and Tyr421, or a combination thereof” recited in claim 1 can be found, for example, in the specification on p. 11, l. 7-9, p. 26, l. 19, and p. 29, ll. 3-4. Support for the term “Figures 1A, B, C, D” can be found in the Figures 1A-D. Support for the term “Lys362, Met363, Arg364, Tyr365, Glu366, His367, Thr416, Arg418, and Tyr421, or a combination thereof” recited in claim 6 can be found, for example, in the specification on p. 12, Table 2.

Claim 10 has been canceled without prejudice.

Claims 41-46 have been added. Support for the new claim 41 can be found in the specification on p. 10, ll. 27-30. Support for the new claim 42 can be found, for example, in the specification on p. 11, ll. 27-29. Support for new claim 43 can be found, for example, in the specification on p. 10, ll. 29-30. Support for the new claim 44 can be found, for example, in the specification on p. 10, ll. 31-35, p. 12, Table 2, p. 26, l. 18, p. 27, ll. 1-4, and p. 29, ll. 2-3. Support for the new claim 45 can be found, for example, in the specification on p. 11, l. 7-9, p. 12, Table 2, p. 26, l. 19, and p. 29, ll. 3-4. Support for the new claim 46 can be found, for example, in the specification on p. 10, ll. 23-30, p. 11, ll. 21-29, p. 12, Table 2, p. 26, l. 19, and p. 29, ll. 3-4.

No new matter has been added by these amendments. Upon entry of the amendments, claims 1, 2, 4-6, 8, 11-34, and 36-46 will be pending. Because claims 12-31 have been withdrawn from consideration, only claims 1, 2, 4-6, 8, 11-34, and 36-46 are at issue.

III. Rejection Of The Claims Under 35 U.S.C. § 112, ¶ 1

Claims 1 and 32 stand rejected under 35 U.S.C. § 112, ¶ 1, as failing to comply with the written description requirement. The Examiner contends that the claims contain subject matter that is not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventors had possession of the claimed invention at the time the application was filed. With regard to claim 1, the Examiner alleges that recitation of the term “having a surface representation and alignment to a structure” is not sufficiently supported by the specification. With regard to claim 32, the Examiner contends that the term “having a spatial configuration according to Figure 1C” is new matter in the claim since the specification fails to provide proper support for this language.

As the terms “having a surface representation and alignment to a structure” and “having a spatial configuration according to Figure 1C” have been deleted, the rejections with respect to these terms are rendered moot.

With regard to claim 1, the Examiner further alleges that recitation of the term “human” is not sufficiently supported by the specification and constitutes new matter.

The Examiner also rejects claims 1-2, 4-6, 8, 10-11, 32-34, and 36-40 under 35 U.S.C. § 112, ¶ 1, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. The Examiner maintains that the specification provides no sequence data that might allow one to characterize the claimed domain as human, mouse or another species.

Applicants respectfully submit that the specification provides clear disclosure and support for human D4 β_c . Based on this disclosure, one having ordinary skill in the art at the time of invention would have readily understood that the claimed cytokine-binding domain of D4 β_c is of human origin.

The human origin of the claimed β_c chain of a cytokine receptor selected from GM-CSF receptor, IL-3 receptor, and IL-5 receptor is referred to consistently throughout the specification. On page 1, lines 12-14, the specification provides that “the common β chain (β_c) of the human granulocyte-macrophage colony-stimulating factor GM-CSF, interleukin-3 (IL-3) and IL-5 receptors” exemplifies heterodimeric cytokine receptors able to recognize several cytokines and transduce their signals (*see* specification, p. 1, ll. 8-13).

It would be also understood by one having ordinary skill in the art that the Applicants worked with isolated and expressed D4 β_c of human origin based on their development of a D4 β_c -specific monoclonal antibody (Mab) effectively inhibiting the high affinity binding of GM-CSF, IL-3, and IL-5 to human eosinophils (*see, e.g.*, specification, p. 2, ll. 10-14, and Example 1, pp. 21-23, ll. 10-18).

Furthermore, all D4 β_c amino acid residues recited in Table 2 at p. 12 of the specification are present in the corresponding positions only within the human β_c chain but not within the β_c chains of cytokine receptors from other species. As shown in the sequence alignment (attached as **Exhibit A**) comparing the sequences of human, mouse, rat, guinea pig, bovine and canine D4 β_c , many of the amino acid residues present in the human D4 β_c and specifically recited in claim 1 (*i.e.*, Arg36, Glu366, His367, Thr416, and Arg418) are poorly conserved among other species.

At the time of the invention, the sequence for the common β_c chain of the human cytokine receptor selected from the group consisting of GM-CSF receptor, IL-3 receptor, and IL-5 was well-known by those in the art and was readily available (*see, e.g.*, the corresponding European Bioinformatics Institute database entry dated August 1, 1991, attached as **Exhibit B** and the 1990 reference by Hayashida et al., *Molecular cloning of a second subunit of the receptor for human granulocyte-macrophage colony-stimulating factor (GM-CSF): reconstitution of a high-affinity GM-CSF receptor*, PROC. NATL. ACAD. SCI. U.S.A. 87:9655-9659 (1990), attached as **Exhibit C**). Accordingly, the specific amino acid residues recited in the present claims and disclosed in Table 2 on page 12 of the specification would have been easily recognized by one having ordinary skill in the art as being specific to the human β_c chain only.

Thus, Applicants submit that the term “human” as recited in claim 1 is well supported by the present specification and does not constitute new matter.

With respect to claims 1-2, 4-6, 8, 10-11, 32-34, and 36-40, the Examiner also maintains that these claims lack of written description under 35 U.S.C. § 112, ¶ 1. Specifically, the Examiner contends that because “the structure of the cytokine-binding domain being claimed is not described . . . [t]he skilled artisan cannot envision the detailed structure of the encompassed domain molecules and therefore conception is not achieved until reduction to practice has occurred [that is,] [t]he nucleic acid itself is required” (Office Action, dated June 6, 2005, pp. 6-7, overlapping paragraph). The Examiner further contends that “the issue here is that the specification fails to recite . . . the specific amino acids that form the portion of the D4β_c chain of the cytokine receptor” (Office Action, dated November 18, 2005, pp. 4-5, overlapping paragraph).

Applicants respectfully traverse the rejection and submit that, based on the combination of structural details and sequence information provided in the specification and figures, one of ordinary skill in the art at the time of the invention would have understood that Applicants were in possession of the claimed domain structure of D4β_c.

With regard to the Examiner’s contention that the specification fails to recite the specific amino acids that form the claimed domain structure of D4β_c of the cytokine receptor, Applicants submit that claim 1 has been amended to recite specific amino acids located in the claimed cytokine-binding domain. Specifically, claim 1 has been amended to recite that the claimed domain comprises

“a portion of the B'-C' loop of D4β_c comprising any one of the following residues Lys362, Met363, Arg364, Tyr365, Glu366, and His367, or a combination thereof, and a groove defined by the B'-C', the F'-G' loops comprising any one of the following residues Thr416, Arg418, and Tyr421, or a combination thereof, and an N-terminal section of D4β_c, said B'-C' loop, F'-G' loop, groove, and N-terminal section having the structure as shown in Figures 1A, B, C, and D.”

The residues of the domain recited in claim 1 are particularly useful as it is these amino acid residues which are functionally important for interacting with cytokines or monoclonal antibodies via van der Waal contact (*see, e.g.*, specification, p. 12, Table 2, col. 3). The identification of these particular amino acid residues and their structural positioning within D4 β_c (recited in the claim by reference to the structure shown in Figures 1A, B, C, and D, and by reference to specific structural elements such as the B'-C' loop, F'-G' loop, groove, and N-terminal section) having particular importance in connection with cytokine-binding was achieved by the present Applicants upon their isolation and crystallization of D4 β_c and could not have been otherwise obtained with certainty.

The specification and figures demonstrate exactly where the claimed structural elements and amino residues are in relation to the domain 4 structure and the β_c chain as a whole (specification, p. 29, ll. 2-4; Figs. 1, 2, and 5A; *see* specification, pp. 9-13, ll. 1-20; pp. 26-27, ll. 10-5; and p. 29, ll. 2-4). For example, the claimed domain is "defined by a portion of a B'-C' loop and a groove defined by B'-C', F'-G' loops and an N-terminal section of D4 β_c chain" (specification, p. 13, ll. 33-35). The "binding domain may be described as a 'groove' comprising a concave surface formed largely, but not exclusively by hydrophobic residues" (specification, p. 11, ll. 27-29). Thus, the claimed loop and groove structures in the pending claims correspond to the cytokine-binding domain as described in detail throughout the specification and specifically on pp. 9-12, ll. 1-20 and in Figures 1-5 and 7.

Therefore, one of ordinary skill in the art would have understood that Applicants possessed the claimed invention. Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. § 112, ¶ 1 be withdrawn.

IV. Rejection Of The Claims Under 35 U.S.C. § 112, ¶ 2

Claims 1-2, 4-6, 8, 10-11, 32-34, and 36-40 stand rejected under 35 U.S.C. § 112, ¶ 2 as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as their invention. In particular, the Examiner contends that the claims are indefinite since a sequence listing is not recited in the claims. Further, the Examiner asserts that

despite previous amendments to claim 1, that the claims remain vague and indefinite because it is unclear which cytokine receptor is being referred to and that there will probably not be a Tyrosine at position 421 in all cytokine receptors. Additionally, with regard to claim 1, the Examiner contends that “(D4 β_c)” should be recited after “Domain 4 of a β_c ” and that “the N-terminal section” lacks sufficient antecedent basis. With regard to claim 4, the Examiner contends that the capitalization of “tyrosine” is not consistent throughout the claims and the term “including” renders the claim indefinite.

The present claims have been amended to correct the formal defects pointed out by the Examiner. Specifically, claim 1 has been amended to recite “(D4 β_c)” after “Domain 4 of a β_c ” and provide antecedent basis for “the N-terminal section.” Claim 4 has been amended to delete the term “including” and to correct for the inconsistent capitalization of the word “tyrosine” in accordance with the Examiner’s direction.

The substantive rejections with respect to the species origin of the claimed domain and the lack of a sequence listing are respectfully traversed.

As discussed above, the specification provides adequate written description and enablement that the claimed domain is of human origin. In fact, claim 1 has been amended to recite several critical amino acid residues which are characteristic of the human sequence (*see* previous section for details). At the time of the invention, the sequence for the common β_c chain of the human cytokine receptor selected from the group consisting of GM-CSF receptor, IL-3 receptor, and IL-5 was well-known by those in the art and was readily available (*see, e.g.,* the corresponding European Bioinformatics Institute database entry dated August 1, 1991, attached as **Exhibit B** and the 1990 reference by Hayashida et al., *Molecular cloning of a second subunit of the receptor for human granulocyte-macrophage colony-stimulating factor (GM-CSF): reconstitution of a high-affinity GM-CSF receptor*, PROC. NATL. ACAD. SCI. U.S.A. 87:9655-9659 (1990), attached as **Exhibit C**).

Thus, Applicants are under no obligation to include the known human β_c chain sequence in the body of the specification (*Capon v. Eshhar*, 418 F.3d 1349, 1355 and 1358 (Fed. Cir. 2005)

(concluding that the Board of Patent Appeals and Interferences erred in determining “that ‘controlling precedent’ required inclusion in the specification of the complete nucleotide sequence,” stating “[w]hen the prior art includes the nucleotide information, precedent does not set a *per se* rule that the information must be determined afresh”); *see* MPEP § 2163, subsections 2 and 3(a) (referring to *Hybridtech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1379-80 and 1384 (Fed. Cir. 1986)) for the proposition that “[i]nformation which is well known in the art need not be described in detail in the specification” and that “[t]he description need only describe in detail that which is new or not conventional”). Accordingly, the claims are definite.

With regard to the Examiner’s contention that it is unclear which cytokine receptor is referred to in claim 1, without conceding as to the correctness of the Examiner’s rejection, Applicants note that claim 1 has been amended to recite that the claimed cytokine-binding domain is derived from “ β_c chain of a human cytokine receptor selected from the group consisting of GM-CSF receptor, IL-3 receptor, and IL-5 receptor.” As specified in the Background Section of the specification, GM-CSF receptor, IL-3 receptor, and IL-5 receptor all act through a common β_c chain subunit (specification, p. 2, ll. 1-3; *see also*, Bagley et al., *The Structural and Functional Basis of Cytokine Receptor Activation: Lessons From the Common β Subunit of the Granulocyte-Macrophage Colony-Stimulating Factor, Interleukin-3 (IL-3), and IL-5 Receptors*, Blood, 89(5):1471-82 (1997), attached as **Exhibit D**).

In light of the above amendments and remarks, the indefiniteness rejections under 35 U.S.C. § 112, ¶ 2 are believed to be overcome and withdrawal of such is kindly requested.

V. Rejection Of The Claims Under 35 U.S.C. § 102(b)

1. WO 97/07215

Claims 1-2, 4-6, 10-11, 32-34, 36-40 stand rejected as being anticipated by the PCT Publication No. WO 97/07215. Specifically, the Examiner contends that the reference discloses an isolated β_c chain particularly Domain 4 of the β_c chain of the IL-3 receptor which binds to at least one cytokine selected from GM-CSF, IL-3 and IL-5” (p. 1, ll. 15-35; p. 4, ll. 37-39; and p. 5, l. 1)

(Office Action, dated June 6, 2005, p. 9, first full paragraph). Thus, the Examiner reasons that the disclosure in the reference “meets the limitations of the instant claims because the reference discloses an isolated cytokine-binding domain of the β_c chain of human IL-3 receptor,” and that, furthermore, “absence of the recitation of an amino acid sequence with respect to the recited residues and in the absence of a specific cytokine-binding domain of specific amino acid residues defined in the claims, the reference anticipates the claims because the metes and the bounds of the cytokine-binding domain being claimed are unclear” (Office Action, dated June 6, 2005, p. 9, second full paragraph).

The Examiner further contends that the claim term “‘portion’ encompasses the entire cytokine-binding domain less a single amino acid at the end of the domain” such that the “metes and bounds of ‘cytokine-binding domain’ are unclear because of the term ‘comprises’ in claim 2, ‘including’ in claim 3 and ‘or combination thereof’ in claim 6, and ‘comprising/comprises’ in claims 32-34, 36-38” (Office Action, dated November 18, 2005, pp. 6-7, overlapping paragraph).

Applicants respectfully traverse the rejection, and request reconsideration.

The present claims call for “[a]n isolated cytokine-binding domain of Domain 4 of a β_c chain (D4 β_c) of a human cytokine receptor.”

The present application provides the first description of the isolation and crystallization of the D4 β_c (specification, p. 8, ll. 25-27, p. 21-23, Example 1). The isolation and crystallization of the D4 β_c (in complex with anti-D4 β_c monoclonal antibody BION-1 developed by the applicants), allowed the present Applicants to determine the structure of the claimed cytokine-binding domain of D4 β_c (*see, e.g.*, specification, p. 8, ll. 29-31; pp. 9-13, ll. 1-20; Figures 1-5 and 7). Thus, without having first isolated and expressed D4 β_c as a discrete entity, one of ordinary skill in the art would not have been able to achieve Applicants’ presently claimed invention.

In contrast to the present invention, WO 97/07215 does not disclose or suggest use of the isolated D4 β_c , nor any specific domain structure thereof involved in cytokine-binding. Instead, WO 97/07215 is concerned with developing a model of activation of human β_c chain generally by

identifying agonists to D4 β_c , and is directed to methods of “activating” the receptor without cytokine-binding (see WO 97/07215, Abstract, pp. 4-5, ll. 37-1; Figs. 4 and 12). WO 97/07215 achieves this either by making activating mutations or by using small molecules which can interact with D4 β_c as agonists to activate the receptor (‘215, p. 5, ll. 4-7; p. 6, ll. 14-16; pp. 11-14, Example 1, Fig. 4). The Examples in WO 97/07215 describe various mutants of human β_c and “mutated” β_c fragments, and although truncated mutants of β_c were made, no mutant consisting solely of the D4 β_c chain is disclosed or suggested (WO 97/07215, pp. 14-16, Example 2).

Most importantly, WO 97/07215 teaches away from the present invention by providing an incorrect predicted structure of D4 β_c . Although WO 97/07215 refers to the modeled structure of D4 β_c and contains a description of the modeling of D4 β_c structure, this was not done by the X-ray crystallography of D4 β_c itself as disclosed in the present application, but rather was deduced on the basis of the crystal coordinates of a different protein, a growth hormone binding protein (GHBP) (WO 97/07215, p. 11, ll. 1-7, Fig. 5). Specifically, in WO 97/07215, the β_c chain sequence was aligned with domain 2 of GHBP and computer modeling was used to predict the structure of the D4 β_c (WO 97/07215, pp. 8-9, ll. 35-2, Fig. 5). Such an indirect method produces only an approximation of the D4 β_c structure, which is different from the D4 β_c cytokine binding domain structure disclosed and claimed in the present application. Indeed, the “groove” identified and determined by the Applicants as part of D4 β_c is absent from the predicted structure shown in Figure 5 of WO 97/07215.

Thus, WO 97/07215 does not disclose or suggest “an isolated cytokine-binding domain of Domain 4 (D4 β_c) of a β_c chain of a human cytokine receptor selected from the group consisting of GM-CSF receptor, IL-3 receptor, and IL-5 receptor” and its structure as recited in the present claims. Accordingly, WO 97/07215 does not anticipate the presently claimed invention and Applicants respectfully request that the Examiner withdraw the anticipation rejection based on this reference.

2. Woodcock et al.

Claims 1-2, 4-6, 10-11, 32-34, 36-40 stand rejected under 35 U.S.C. § 102(b) as being anticipated by Woodcock et al., *The Human Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) Receptor Exists as a Preformed Receptor Complex That Can Be Activated by GM-CSF, Interleukin-3, or Interleukin-5*, Blood, 90(8):3005-17 (1997) (Woodcock). Specifically, the Examiner contends that the reference discloses “using specific antibodies which immunoprecipitate the β_c chain of the IL-3 receptor (β_4 Domain) which binds to at least one cytokine selected from GM-CSF, IL-3 and IL-5 and for affinity purification of the β_c region” (Woodcock, abstract, p. 3005, col. 2, ll. 6-11, p. 3006, col. 2, paragraph disclosing “antibodies”) (Office Action, dated June 6, 2005, p. 9, third full paragraph).

The Examiner further contends that “in the absence of a definition for ‘a portion’ in the instant specification, ‘a portion’ encompasses the entire cytokine-binding domain less a single amino acid at the end of the domain” such that the “metes and bounds of ‘cytokine-binding domain’ are unclear because of the term ‘comprises’ in claim 2, ‘including’ in claim 3 and ‘or combination thereof’ in claim 6, and ‘comprising/comprises’ in claims 32-34, 36-38” (Office Action, dated November 18, 2005, p. 7, second full paragraph).

Applicants respectfully traverse this rejection, and request reconsideration.

The present claims call for “[a]n isolated cytokine-binding domain of Domain 4 of a β_c chain (D4 β_c) of a human cytokine receptor.”

Applicants respectfully submit that the present invention distinguishes over Woodcock for at least those reasons noted above in relation to WO 97/07215. That is, similar to WO 97/07215, Woodcock does not disclose or suggest isolation of D4 β_c .

Woodcock discloses the entire β_c as an isolated protein and various monoclonal antibodies against it (Woodcock, Abstract) and is silent in regard to use of D4 β_c as a discrete physical entity, i.e., as an isolated domain, much less the precise cytokine-binding domain structure of D4 β_c . Thus,

one of ordinary skill in the art, upon review of Woodcock, would be no closer to achieving Applicants' presently claimed invention on the basis of its disclosure.

Accordingly, Applicants respectfully request that the Examiner withdraw the anticipation rejection based on the Woodcock reference.

CONCLUSION

Applicants respectfully request entry of the foregoing amendments and remarks in the file history of this application. In view of the above amendments and remarks, it is respectfully submitted that the pending claims are now in condition for allowance and such action is earnestly solicited. If the Examiner believes that a telephone conversation would help advance the prosecution in this case, the Examiner is respectfully requested to call the undersigned attorney at (212) 527-7601. The Examiner is hereby authorized to charge any additional fees associated with this response to our Deposit Account No. 04-0100.

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Respectfully submitted,

By 

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Attorneys/Agents For Applicant

Alignment of β c domain 4

BION-1 contact	*****
Human	IQMAPPSLNVTKDGDSYSLRWETMKMRYEHIDHTFEIQYR
Murine (β_c)	IQMEPPTLNLTKNRDSYSLHWETQKMAYSFIEHTFQVQYK
β strands	-----
	4A 4B 4C
BION-1 contact	*
Human	KDTATWKDSKTETLQNAHSMALPALEPSTRYWARVRVRTS
Murine (β_c)	KKSDSWEDSKTENLDRAHSMDLSQLEPDTSYCARVRVKP-
β strands	-
	4D 4E 4F
BION-1 contact	* *
Human	RTGYNGIWSEWSEARSWDTE
Murine (β_c)	ISNYDGIWSKWSEEYTWKTD
β strands	----
	4G
BION-1 contact	*****
Human	IQMAPPSLNVTKDGDSYSLRWETMKMRYEHIDHTFEIQYR
Murine (β_c)	IQMEPPTLNLTKNRDSYSLHWETQKMAYSFIEHTFQVQYK
Murine (β_{IL-3})	IQMEPPILNQTKNRDSYSLHWETQKIP-KYIDHTFQVQYK
Rat (β_c)	IQMNPPTLNLTKNRDSYSLHWETQKMSYPFIQHAFQVQYK
Guinea Pig	IQMAAPTLNVTKDGDYSLRWVTEKMYYSHIENTFEIQYR
Bovine	IQMAPPTLNVTKGRDGYILHWREEKMSYSHIACIFQVQYK
Canine	IQMARPTLNVTKNRDGYTLHWMAKEMFYKHIGHTFQVQYK
β strands	-----
	4A 4B 4C
BION-1 contact	*
Human	KDTATWKDSKTETLQNAHSMALPALEPSTRYWARVRVRTS
Murine (β_c)	KKSDSWEDSKTENLDRAHSMDLSQLEPDTSYCARVRVKP-
Murine (β_{IL-3})	KKSESWKDSKTENLGRVNSMDLPQLEPDTSYCARVRVKP-
Rat (β_c)	KKLDRWEDSKTENLNHAHSMDLPPLEPGTSYCARVRVKT-
Guinea Pig	TAGDRWENSKTETLKNAHNMPLPPLEPATTYLARVRVKPS
Bovine	KEGASWEDTKTEDFQNAHTMSLPPLEPASRYQARVRVKPD
Canine	KDTSWEKSVMEKLENAHSMSPLEPSTRYQARVRVKPT
β strands	-
	4D 4E 4F
BION-1 contact	* *
Human	RTG-YNGIWSEWSEARSWDTE
Murine (β_c)	ISN-YDGIWSKWSEEYTWKTD
Murine (β_{IL-3})	ISD-YDGIWSEWSNEYTWTDD
Rat (β_c)	IPE-YKGLWSEWSNECTWTDD
Guinea Pig	PGGAYNGIWSEWSEEQRWTDD
Bovine	PGN-YNGIWSEWSEARSWDTE
Canine	RG--YDGVWSEWSEEHFWDTE
β strands	----
	4G

BION-1 directly contacts residues in the B'-C' and F''-G' loops of human β c domain 4 (see table 2). Some of these residues have homologues that are highly conserved in β c across a range of species such as Lys³⁶², Met³⁶³, Tyr³⁶⁵ and Tyr⁴²¹. Other residues are poorly conserved and include Arg³⁶⁴, Glu³⁶⁶, His³⁶⁷, Thr⁴¹⁶ and Arg⁴¹⁸. Importantly, two of these poorly conserved residues, Glu³⁶⁶ and Arg⁴¹⁸, are required for BION-1 binding.

Source of sequence used for alignment;

Human β_c	>gb AAH70085.1 CSF2RB protein [Homo sapiens]
Murine β_c	>gb AAL90771.1 interleukin 3 receptor-like protein [Mus musculus]
Murine β_{IL-3}	>gi 417183 sp P26954 IL3B2_MOUSE Interleukin-3 receptor class 2 beta chain precursor (Interleukin-3 receptor class II beta chain) (Colony-stimulating factor 2 receptor, beta 2 chain)
Rat β_c	>gb AAB35068.1 interleukin-3 receptor beta-subunit; rIL-3R beta [Rattus sp.] ref NP_598239.1 colony stimulating factor 2 receptor, beta 1, low-affinity (granulocyte-macrophage) [Rattus norvegicus]
Guinea Pig β_c	>gb AAC77520.1 interleukin-5 receptor beta chain [Cavia porcellus]
Bovine β_c	>ref XP_606956.2 PREDICTED: similar to Cytokine receptor common beta chain precursor (GM-CSF/IL-3/IL-5 receptor common beta-chain) (CD131 antigen) (CDw131 antigen) [Bos taurus]
Canine β_c	>ref XP_538397.2 PREDICTED: similar to Cytokine receptor common beta chain precursor (GM-CSF/IL-3/IL-5 receptor common beta-chain) (CD131 antigen) (CDw131 antigen) [Canis familiaris]



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DATABASE BROWSING

EBI Dbfetch

ID HSGMCS01 standard; mRNA; HUM; 2996 BP.
 XX
 AC M59941; M38275;
 XX
 SV M59941.1
 XX
 DT 01-AUG-1991 (Rel. 28, Created)
 DT 01-JUL-1999 (Rel. 60, Last updated, Version 3)
 XX
 DE Human GM-CSF receptor beta chain mRNA, complete cds.
 XX
 KW cytokine receptor; GM-CSF receptor; growth factor receptor;
 KW lymphokine receptor.
 XX
 OS Homo sapiens (human)
 OC Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia;
 OC Eutheria; Euarchontoglires; Primates; Catarrhini; Hominidae; Homo.
 XX
 RN [1]
 RP 1-2996
 RX PUBMED; 1702217.
 RA Hayashida K., Kitamura T., Gorman D.M., Arai K., Yokota T., Miyajima A.;
 RT "Molecular cloning of a second subunit of the receptor for human
 RT granulocyte-macrophage colony-stimulating factor (GM-CSF): reconstitution
 RT of a high-affinity GM-CSF receptor";
 RL Proc. Natl. Acad. Sci. U.S.A. 87(24):9655-9659(1990).
 XX
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 RP 1-2996
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 RT ;
 RL Submitted (06-FEB-1991) to the EMBL/GenBank/DDBJ databases.
 RL Toshio Kitamura, Department of Molecular Biology, DNAX Research Institute
 RL of Molecular and Cellular Biology, 901 California Avenue, Palo Alto, CA
 RL 94304-1104, USA
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Molecular cloning of a second subunit of the receptor for human granulocyte-macrophage colony-stimulating factor (GM-CSF): Reconstitution of a high-affinity GM-CSF receptor

(cytokine receptor/hemopoietic growth factor/hemopoiesis gene family)

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ABSTRACT Using the mouse interleukin 3 (IL-3) receptor cDNA as a probe, we obtained a homologous cDNA (KH97) from a cDNA library of a human hemopoietic cell line, TF-1. The protein encoded by the KH97 cDNA has 56% amino acid sequence identity with the mouse IL-3 receptor and retains features common to the family of cytokine receptors. Fibroblasts transfected with the KH97 cDNA expressed a protein of 120 kDa but did not bind any human cytokines, including IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF). Interestingly, cotransfection of cDNAs for KH97 and the low-affinity human GM-CSF receptor in fibroblasts resulted in formation of a high-affinity receptor for GM-CSF. The dissociation rate of GM-CSF from the reconstituted high-affinity receptor was slower than that from the low-affinity site, whereas the association rate was unchanged. Cross-linking of ¹²⁵I-labeled GM-CSF to fibroblasts cotransfected with both cDNAs revealed the same cross-linking patterns as in TF-1 cells—i.e., two major proteins of 80 and 120 kDa which correspond to the low-affinity GM-CSF receptor and the KH97 protein, respectively. These results indicate that the high-affinity GM-CSF receptor is composed of at least two components in a manner analogous to the IL-2 receptor. We therefore propose to designate the low-affinity GM-CSF receptor and the KH97 protein as the α and β subunits of the GM-CSF receptor, respectively.

All hemopoietic cells ultimately arise from self-renewing pluripotent hemopoietic stem cells which are produced continuously in the bone marrow. Bone marrow stromal cells and a number of soluble factors, known as cytokines, play crucial roles in this process. Among these cytokines, interleukin 3 (IL-3), also known as multi-colony-stimulating factor (multi-CSF), stimulates early progenitor cells and supports the development of various cell lineages (1). While GM-CSF was initially defined as a factor that gives rise to granulocyte and macrophage colonies *in vitro*, recent evidence indicates that GM-CSF has broader biological activities, including stimulation of early hemopoietic progenitor cells and the development of other cell lineages (2, 3).

The biological effects of both GM-CSF and IL-3 are mediated by specific cell surface receptors. The human GM-CSF (hGM-CSF) receptor, cloned by Gearing *et al.* (4), exhibits low-affinity binding for GM-CSF when expressed on COS7 cells. Although there is evidence indicating that GM-CSF induces tyrosine phosphorylation (5), no tyrosine kinase consensus sequence was found (4). It is likely that the functional high-affinity GM-CSF receptor is composed of multiple subunits. Both GM-CSF and IL-3 induce tyrosine

phosphorylation of a similar set of proteins (6–8) and they have overlapping biological activities (1–3). In addition, evidence indicates that the binding of hGM-CSF to its receptor is partially inhibited by human IL-3 (hIL-3) and vice versa (9–11). These results suggest that the hGM-CSF receptor and the hIL-3 receptor may share a common component.

Mouse IL-3 (mIL-3)-responsive cells express low- and high-affinity receptors for IL-3 (12–14). We recently isolated a cDNA (AIC2A) encoding a low-affinity mIL-3 binding protein which is a member of a recently identified cytokine receptor family (15). Although AIC2A does not contain a tyrosine kinase consensus sequence, AIC2A is a component of the high-affinity receptor (J. Schreurs and A.M., unpublished results). We also isolated a cDNA (AIC2B) which is highly identical (95% at the nucleotide level) to the IL-3 receptor cDNA (AIC2A) but is derived from a distinct gene (16). Despite its unusually high sequence identity with the IL-3 receptor, the AIC2B protein does not bind IL-3 and its function is currently unknown.

In this report, we present the cloning of a human cDNA which has homology with the mIL-3 receptor cDNA. The protein encoded by the cloned cDNA alone did not bind any of the cytokines tested. However, it conferred high-affinity binding for hGM-CSF when cotransfected with the low-affinity hGM-CSF receptor cDNA. This result indicates that the cloned IL-3 receptor-like cDNA (KH97) encodes a second subunit of the high-affinity hGM-CSF receptor.

MATERIALS AND METHODS

Construction of cDNA Library and Isolation of cDNA Clones. Poly(A)⁺ RNA isolated from TF-1 cells (17) was converted to double-stranded cDNA by using oligo(dT) primers or specific primers corresponding to the cDNA sequence (Fig. 1). cDNA libraries were constructed either in the λ gt11 phage vector or the simian virus 40-based mammalian expression vector pME18 (K. Maruyama and A.M., unpublished results). Using a ³²P-labeled mouse IL-3 receptor cDNA fragment (15) as a hybridization probe, we isolated a 3-kilobase (kb) human cDNA fragment (KH85) from the phage library under low-stringency conditions: hybridization at 42°C with 6× SSPE (1× SSPE is 150 mM NaCl/100 mM NaH₂PO₄/1 mM EDTA, pH 7.4) in the presence of 20% (vol/vol) formamide and washing at 50°C with 2× SSPE.

Abbreviations: IL-2, IL-3, etc., interleukin 2, interleukin 3, etc.; GM-CSF, granulocyte-macrophage colony-stimulating factor; h-, human; m-, mouse.

‡To whom reprint requests should be addressed.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M38275).

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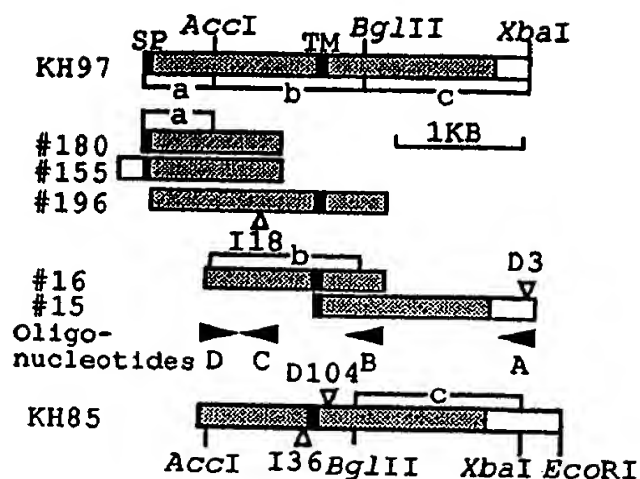


FIG. 1. The cloned cDNA fragments. Typical cDNA fragments obtained by using specific oligonucleotide primers are shown as bars, with the unshaded portion indicating the noncoding regions. The locations of oligonucleotides used to prime the cDNA synthesis are shown as arrowheads A, B, and C. An oligonucleotide corresponding to the region D and the KH85 cDNA fragment were used to isolate these cDNA fragments. All the cloned DNA fragments had the same sequence except for small insertions and deletions as indicated by I18 (an 18-base-pair insertion), I36 (a 36-base-pair insertion), D104 (a 104-base-pair deletion), and D3 (a 3-base-pair deletion). The KH97 cDNA was constructed with #180 (fragment a), #16 (fragment b), and KH85 (fragment c) as indicated. SP and TM indicate the signal peptide and the transmembrane domain, respectively. Scale indicates 1 kilobase.

The cDNA encoding the low-affinity hGM-CSF receptor was isolated by using the polymerase chain reaction primed with specific oligonucleotides corresponding to the 5' untranslated and the 3' untranslated regions of the published sequence (4). The cloned DNA fragment was sequenced to confirm the identity of the cDNA.

Transfection. Five micrograms of either individual plasmid DNA or a combination of two plasmid DNAs was transfected into semiconfluent COS-7 cells (African green monkey kidney cells expressing the T antigen of simian virus 40) by the DEAE-dextran method as described previously (18). Three days after transfection, COS-7 cells were harvested and analyzed by ligand binding assays or chemical cross-linking

experiments. NIH 3T3 mouse cells were stably transfected, using the *neo* gene as a selection marker, by the calcium phosphate procedure (19). Stable transfectants were selected with G418 at 1 mg/ml.

Radioiodination of hGM-CSF and Binding Experiments. *Escherichia coli*-derived hGM-CSF iodinated with Bolton-Hunter reagent (125 I-GM-CSF) was used for binding assays as described previously (20). Dissociation constants were obtained by the LIGAND program (21). Chemical cross-linking was performed with 0.2 mM disuccinimidyl suberate on transfected COS-7 cells (10^6 cells) or NIH 3T3 stable transfectants (3×10^6 cells) preincubated with 4 nM 125 I-GM-CSF. Proteins were analyzed as described previously (20).

RESULTS

Isolation and Characterization of a Human cDNA Homologous to the mIL-3 Receptor cDNA. Using the mIL-3 receptor cDNA as a probe, we screened a cDNA library made from a human erythroleukemic cell line, TF-1, which responds to multiple human factors, including IL-3, IL-4, IL-5, GM-CSF, and erythropoietin (17). A cDNA clone (KH85) homologous to the mIL-3 receptor cDNA (approximately 70% identical at the nucleotide level) was obtained from about 4×10^5 independent clones. This clone lacks about 600 bases from its 5' end compared with the sequence of the mouse cDNA. We therefore prepared cDNA libraries by using specific primers based on the KH85 sequence and screened these libraries with the KH85 probes (Fig. 1). We found only one type of cDNA among 26 positive clones analyzed. Although several cDNAs with an insertion and/or a deletion were isolated (Fig. 1), these cDNAs seemed to be created by alternative splicing rather than encoded by a distinct gene, because the insertions and deletions were found at sites corresponding to the exon-intron junctions of the mouse AIC2 genes (D.M.G., unpublished results). We reconstructed a cDNA (KH97) encoding the entire protein (Fig. 1) and used this for further studies.

Comparison of the amino acid sequence encoded by the KH97 cDNA with that of the AIC2A and AIC2B proteins showed 56% and 55% identity, respectively (Fig. 2). The homology was distributed throughout the coding region in

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AIC2A	1	MDQQ*ATW*CY**V***GHEVTEE**V**K**E*****NR**I**S***E***G*I**M**LYHQLDKI	QS***E**EKIM**E**SSH*****YTR*SN	
AIC2B	1	MDQQ*ATW*CY**V***GHEVTEE**V**K**E*****NR**I**S***E***G*I**M**YHQL*KK	Q***E**EKIM**E**SSH*****YTR*SI	
	106	TDVDYFSFQDRPLGTRLTTLTQHVQPPPEPRDLQISTDQDHFLTLWSVALGSPQSHWLSPGDLEFEVVKRLQDSWEDAAILLNNTSQATLGPENLMP	SSSTYVARVRTRLAPGSRLSGR	
	110	G*N**Y*****D**IQ*M**P**A*****P*K*I**H**PSG***E***S**DS*VS***SK*I*****SS*HTSNF*VN**E**KLFL*N*I**A*****SA**S***		
	109	*NE**Y**R**SD**IQ*M**P**A**N*****L*KNVS**SSE*R***E***S**DA*VS***SK*I*****A*****YS*HTSKF*VNF*KLFL*N*I**A*****Y**S***		
	226	PSKNSPEVCWDSQPGDEAQPQNLFFDGAAVLSWEVRKEVASSVSFGLFYKPSPDAGEEEFSPVLRGLG	SLHTRHH	IPVDPATHGQYIVSVQPRRAEKHKISSVNIOMAPPS
	230	**R**H*****K*****IQS*H*****WTQTG*****R**A**P**K***VK*PQ	A*VY**YF*SL**E*SA*S**T**KHLEQG*F*M*YH**E**I	
	229	**R**H*****K*****IQS*H*****WTQTG*****R**V**P**K***VK*PP*A*VY**YF*SL**E*SA*S**T**KHLEQG*F*M*YH**E**I		
	345	LVNTKQDYSYSLRWETMKMYEHIDHTFEIQYRKDTATWDSKTETLQNAHSMALPALEPSTRYWARVRVTSRTGYNGINSEWS	SEARSWDTES	LPMVVLALIVIFLTTAVLLALRFQ
	349	**Q**NR*****H**Q*IP	KY**Q**K**KSES*****N*GRVN**D**Q**D*S*C*****KPI	SN*D**K**EYTK*DW*M*TLNIV**LV**ITL**I**G
	349	**L**NR*****H**Q*A*SF*E**QV**K*KSDS*E*****N*DR**D**SQ**D*S*C*****KPI	SN*D**K**EYTK*DW*M*TLNIV**LV**ITL**I**G	
	465	TYGYRLRRKWEKIPNPSKSHLPQNGSAELNPPGMSAFTSGSPPHQCPWGSRFPELEGVFPVGFQDSEVSPLTIEDPKHVCDP	PSGPDTPAASDLPTQPPSPQPGPFAASHTPEKQA	
	467	V*****TY**K*****L**D*GKG*****A**ATKN*AL**QSRLLA*QQ*SYEHLE*NN*****NIIR*****SES**L*NV*VEG*IP*SR*R**L		
	468	VSV**TY**K*****L**D*GKG*****A**ATKN*AL**QSRLLA*QQ*ESYAHLE*NN*****NIIR*****SES**L*NV*VEG*IP*SR*R**L		
	585	SSFDNFGPYLGPSPHRSRSLPDILQPEPPQEGGSQKSPPGSLEYLCLPAGQVOLVPLAQAMGPGQAVEVERRP	SQGAAGSP	SLESGGGPAPPALGPRVGGQDQKDSIPAIPMSGDTED
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	587	P*****Q*H*****LPD*LGS**V**L*PAL*****M**P*****S*V**Q**MD*QCGS*LETT**V*PKEN	*PVELSMEE*EAR*N*MTL*I**GP*G	
	705	PGVASGYVSSADLVFTPNSSGASSVSLVPSLGLPDSQTPSLCPGLASGPPGAPGPVKSGFEGYVELPPIEGRSRSPRNNPVP	PEAKSPVLNPGERPADVSPTSPOPEGLLVLOQVQDYCF	
	704	SMM**D**TPG*P*L*LPT*PL*T**G*****A*S*****LK*PRV*S*S*ALGPP**D*****SVSQAAT**PGH*A*V*S**TVI**PREE*G*A**H*****R*****		
	704	SMM**D**TPG*P*L*LPT*PL*T**G*****A*S*****LK*PRV*S*S*ALGPP**D*****SVSQAAT**PGH*A*V*S**TVI**PREE*G*A**H*****R*****		
	825	LPGLGPGPLSLRSPKSSPGPGPEIKNLDAFQVKKPPGQAVPQVPIQLFKALKQDDYLSLPPWEVKNKPEVC	897	
	824	*****S**PH**P**SLCS*TED**DLS**F*Y*PL**A**A**F**S**Y	878	
	824	*****S**PH**P**SLCS*TED**V*DL**F*Y*PM**A**A**F**S**H*****DNSQS*K**	896	

FIG. 2. Comparison of amino acid sequence of KH97 with the mouse AIC2A and AIC2B proteins. Asterisks on the mouse AIC2A and AIC2B sequences indicate the same amino acid as that of the human KH97 sequence. The signal sequences and the transmembrane domains are shown by boxes. The conserved cysteine residues and the WSXWS motif of the cytokine receptor family are also indicated by boxes. The WSXWS-like sequence is indicated by the dotted outlines. Potential N-linked glycosylation sites are marked by bars.

both cases. The AIC2B protein has an extra 18 amino acids at the C terminus compared with the mIL-3 receptor (AIC2A), and the KH97 protein also has the extra 18 amino acids (Fig. 2). Because of the high sequence similarity between AIC2A and AIC2B (16), it is not clear to which of the mouse genes the KH97 cDNA corresponds.

The KH97 mRNA was detected in the myelogenous leukemic cell lines TF-1 and KG1 but not in the NK cell line YT or the mouse IL-3- and GM-CSF-dependent cell line PT18 under stringent hybridization conditions (Fig. 3).

Expression of KH97 in COS-7. A transient expression system using COS-7 cells was used to evaluate expression of the KH97 cDNA. Because of its extensive sequence similarity to the mIL-3 receptor cDNA, we examined whether hIL-3 could bind to COS-7 cells transfected with the KH97 cDNA. Using up to 20 nM 125 I-hIL-3, we could not detect any specific binding. We also constructed full-length cDNAs of KH97 variants (Fig. 1), which were presumably derived from alternative splicing, and tested the binding of hIL-3 to transfected COS-7 cells. However, we did not find any specific binding. We then examined the binding of other cytokines. However, hIL-2 (1 nM), hIL-4 (1 nM), hIL-5 (5 nM), hGM-CSF (20 nM), and human erythropoietin (10 nM) all showed no specific binding to KH97-transfected COS-7 cells at the indicated concentrations. To exclude the possibility that COS-7 cells failed to express the protein encoded by the KH97 cDNA, we prepared antibodies against a peptide encoded by the KH97 cDNA and used them to detect the KH97 cDNA-encoded protein in COS-7 cells. Western blotting using anti-peptide antibodies confirmed the expression of a 120-kDa protein in the KH97 cDNA-transfected COS-7 cells (data not shown). We therefore concluded that the KH97 protein did not bind any cytokines examined when expressed in COS-7 cells.

Cotransfection of the KH97 cDNA and the GM-CSF Receptor cDNA. Although the KH97 protein did not bind any of the cytokines we examined, there still remained the possibility that it is a component of another known or unknown cytokine receptor. It has been shown that the β chain of the IL-2 receptor does not bind IL-2 when expressed in COS-7 cells, but it does bind IL-2 with intermediate affinity in Jurkat cells (22). Furthermore, coexpression of the low-affinity IL-2 receptor (α chain) and the β chain forms a high-affinity binding site (22). These observations indicate that the binding of a ligand to its receptor can be determined by the interaction of multiple proteins. Combining this idea with the observa-

tion that the IL-3 and GM-CSF receptors may share a common element (9–11), we considered the possibility that the KH97 protein is a subunit of the hGM-CSF receptor. To examine this possibility, we cotransfected the KH97 cDNA with the low-affinity GM-CSF receptor cDNA (4).

We examined the equilibrium binding of hGM-CSF to COS-7 cells transfected with the hGM-CSF receptor cDNA, the KH97 cDNA, or a combination of these cDNAs (Fig. 4). The hGM-CSF receptor expressed exclusively low-affinity binding sites ($K_d = 3.2$ nM) as reported by Gearing *et al.* (4), whereas the KH97 protein alone did not express any detectable binding for GM-CSF. However, cotransfection of these two cDNAs resulted in the expression of both high- (120 pM) and low- (6.6 nM) affinity binding sites.

The same results were obtained with stable transfectants of NIH 3T3 cells (Fig. 4): hGM-CSF receptor cDNA-transfected NIH 3T3 cells bound hGM-CSF with $K_d = 2.7$ nM, whereas the NIH 3T3 transfected with both the hGM-CSF receptor and the KH97 cDNAs bound hGM-CSF with $K_d = 170$ pM. Because of the low expression of the low-affinity hGM-CSF receptor compared with the KH97 protein in this NIH 3T3 transfectant, no statistically significant low-affinity binding site was found by using the LIGAND program (21). Again no specific binding of hGM-CSF was detected in the cells transfected with the KH97 cDNA alone.

In both COS-7 and NIH 3T3 cells, binding of 125 I-hGM-CSF was blocked by hGM-CSF but not by hIL-3. In addition, cotransfection of the KH97 cDNA with the human IL-2 receptor α chain cDNA or the human IL-4 receptor cDNA did not change the binding affinity of their respective ligands. Cotransfection of the cDNAs encoding the low-affinity hGM-CSF receptor with that for the IL-2 receptor β chain, the IL-4 receptor, mouse AIC2A, or AIC2B also did not change the affinity for hGM-CSF. Thus, formation of the high-affinity GM-CSF receptor is specific to the combination of the low-affinity GM-CSF receptor and the KH97 protein.

We then analyzed the binding kinetics to determine whether the high-affinity binding of hGM-CSF to cotransfected cells was due to an increased rate of association or a decreased rate of dissociation. As shown in Fig. 5, GM-CSF

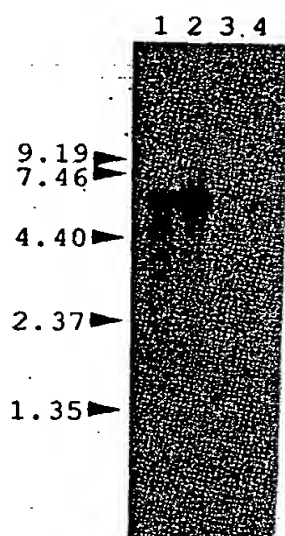


FIG. 3. Northern blotting of poly(A)⁺ RNA from various cell lines. Three micrograms of each poly(A)⁺ RNA was electrophoresed on a 1% agarose gel, transferred to a nitrocellulose membrane, and hybridized with the 32 P-labeled KH97 cDNA. Lane 1, TF-1; lane 2, KG-1; lane 3, YT; and lane 4, PT18. Sizes are given in kb.

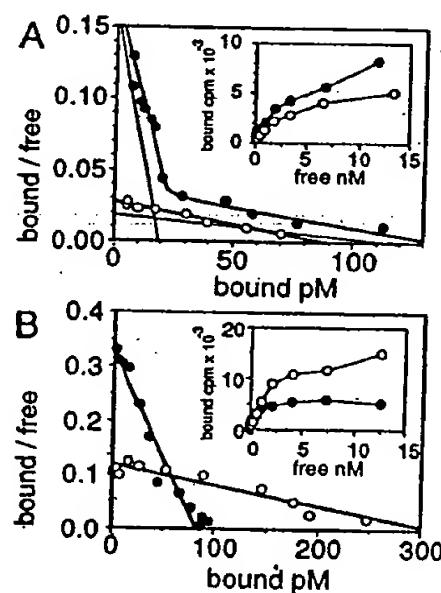


FIG. 4. Binding of 125 I-hGM-CSF. (A) Duplicate suspensions of 2×10^5 COS-7 cells transiently transfected with the hGM-CSF receptor and KH97 cDNAs were used for 125 I-hGM-CSF binding assays. (B) Duplicate suspensions of NIH 3T3 stable transfectants (5×10^6 cells) were used for binding assays. Binding assays were performed at 4°C. ○, Cells transfected with the hGM-CSF receptor cDNA and the vector DNA. ●, Cells cotransfected with the hGM-CSF receptor cDNA and the KH97 cDNA. Scatchard plots of the binding data are shown. (Insets) Equilibrium binding profiles.

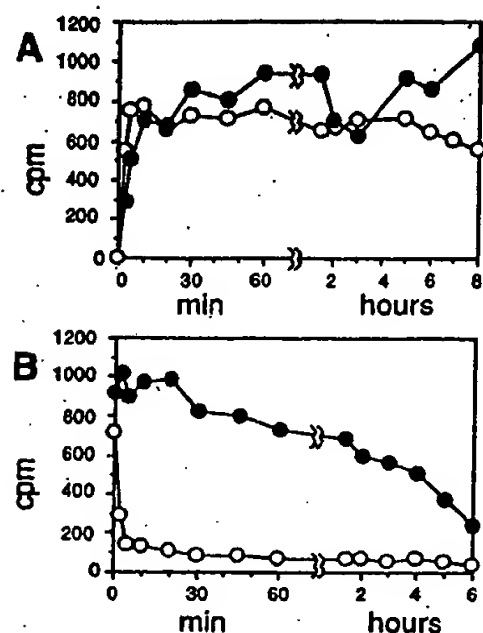


FIG. 5. Kinetics of binding of ¹²⁵I-hGM-CSF to the receptor. (A) Association rate. NIH 3T3 stable transfectants were incubated with 200 pM ¹²⁵I-hGM-CSF at 4°C for various times and the cell-bound radioactivity was measured. (B) Dissociation rate. A 200-fold excess of unlabeled hGM-CSF was added to the NIH 3T3 stable transfectants, which were preincubated with 200 pM ¹²⁵I-hGM-CSF for 4 hr at 4°C, and the residual cell-bound radioactivity was then measured at various times at 4°C. O, NIH 3T3 cells expressing the low-affinity hGM-CSF receptor; ●, NIH 3T3 cells expressing both the hGM-CSF receptor and the KH97 protein.

binding increased with similar kinetics in both hGM-CSF receptor cDNA-transfected cells and cotransfected cells. In dissociation experiments, the addition of unlabeled hGM-CSF to cells preequilibrated with ¹²⁵I-hGM-CSF led to the rapid release of radioligand from the hGM-CSF receptor-transfectants ($t_{1/2} = 2$ min), and a slow release ($t_{1/2} = 290$ min) from the cells expressing both proteins. These results indicate that the high-affinity binding of hGM-CSF to the cotransfected cells is due to the slow dissociation of hGM-CSF from the receptor.

Cross-Linking of hGM-CSF to the KH97 Protein. To test if the KH97 protein actually binds hGM-CSF when coexpressed with the low-affinity hGM-CSF receptor in COS-7 or NIH 3T3 cells, we performed chemical cross-linking experiments using ¹²⁵I-hGM-CSF (Fig. 6). Cross-linking of ¹²⁵I-hGM-CSF to the low-affinity hGM-CSF receptor-expressing cells showed only one band of 95 kDa (Fig. 6B, lane 5), whereas no specific band was detected in cells expressing only the KH97 protein (Fig. 6B, lane 9). However, cross-linking of the cells expressing both proteins revealed three bands, of 95, 135, and 210 kDa (Fig. 6B, lane 6). This cross-linking pattern was identical to that obtained with TF-1 cells (Fig. 6B, lane 10). These bands were not detected when cross-linking was performed in the presence of an excess of unlabeled hGM-CSF (data not shown). Subtraction of the molecular mass of hGM-CSF from the molecular mass of the cross-linked proteins results in calculated masses of 80, 120, and 195 kDa, which correspond, respectively, to the low-affinity hGM-CSF receptor, the KH97 protein, and possibly a complex of the two. Similar results were obtained with NIH 3T3 transfectants (Fig. 6B, lanes 1–4).

Cross-linking of hGM-CSF to the KH97 protein was further confirmed by making cytoplasmic domain deletion mutants of KH97 (Fig. 6A). Deletion mutants of the KH97 cDNA were cotransfected with the low-affinity hGM-CSF receptor cDNA into COS-7 cells. Whereas the band at 95 kDa was not changed, the band at 135 kDa was shifted to lower molecular masses in cells transfected with these deletion mutants (Fig.

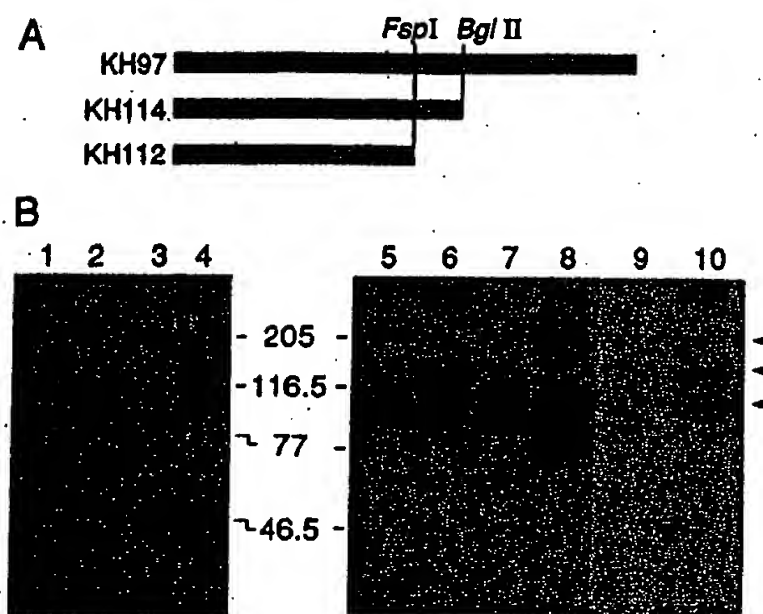


FIG. 6. Expression of GM-CSF receptors on COS-7 cells and NIH 3T3 cells. (A) Structure of the cDNAs encoding the KH97 protein and its deletion mutants. KH114 is truncated at the *Bgl* II site and KH112 is truncated at the *Fsp* I site (Fig. 1). (B) Cross-linking of ¹²⁵I-hGM-CSF to the transfected COS-7 cells or NIH 3T3 stable transfectants. Lanes 1–4, NIH 3T3 cells; lanes 5–9, COS-7 cells. Cells were transfected with the following: lane 1, mock transfection; lane 2, GM-CSF receptor cDNA; lane 3, KH97 cDNA; lane 4, GM-CSF receptor cDNA and KH97 cDNA; lane 5, GM-CSF receptor cDNA; lane 6, GM-CSF receptor cDNA and KH97 cDNA; lane 7, GM-CSF receptor cDNA and KH114 cDNA; lane 8, GM-CSF receptor cDNA and KH112 cDNA; and lane 9, KH97 cDNA. Lane 10, cross-linking with TF-1 cells. Specific bands detected in COS-7 cells transfected with deletion mutants are marked by dots (lanes 6–8). Protein masses are given in kDa.

6, lanes 6–8, marked by dots). The shift of the molecular mass was consistent with the shift of the bands revealed by Western blotting using anti-peptide antibodies against the KH97 protein (data not shown). These results clearly indicate that the KH97 protein is cross-linked with hGM-CSF when coexpressed with the low-affinity hGM-CSF receptor. In addition, this experiment demonstrates that formation of the high-affinity receptor for hGM-CSF does not require the cytoplasmic domain of the KH97 protein.

DISCUSSION

Our results demonstrate that the low-affinity hGM-CSF receptor together with the KH97 protein forms a high-affinity receptor for hGM-CSF. This is analogous to the formation of a high-affinity receptor for IL-2 by coexpression of the α and β chains of the IL-2 receptor. The α chain of the IL-2 receptor binds IL-2 with low affinity (23, 24), whereas the β chain by itself does not bind IL-2 when expressed in COS-7 cells (22). However, coexpression of both chains leads to the formation of a high-affinity receptor, and both chains can be cross-linked with IL-2 (22). Because of similarity between the IL-2 and the hGM-CSF high-affinity receptors, we propose to designate the low-affinity hGM-CSF receptor as the α chain and the KH97 protein as the β chain of the hGM-CSF receptor.

It is of particular interest that the KH97 cDNA was isolated on the basis of its homology to the mIL-3 receptor. Among various cytokines the sequence conservation of IL-3 between mouse and human is unusually weak (only 29% identity at the amino acid level) (25). Nevertheless we were able to isolate a human cDNA which was homologous (56% identity at the amino acid level) to the mIL-3 receptor cDNA. However, as described above, we were unable to demonstrate IL-3 bind-

ing to the KH97 protein expressed on fibroblasts. Recently, we isolated a second mouse gene (AIC2B) which is highly homologous to the mIL-3 receptor gene (AIC2A), but the AIC2B protein does not bind mIL-3 (16). One possibility is that the KH97 protein is the human protein corresponding to the mouse AIC2B protein and therefore cannot bind IL-3. If this is the case, there may exist an AIC2A-like human protein which binds hIL-3. To address this question we have extensively searched for additional cDNA clones which might hybridize with either the mouse AIC2A or the human KH97 cDNA probe. However, we could not find any cDNA that is homologous to, yet distinct from, the KH97 cDNA. Since the abundance of the AIC2B mRNA is generally higher than that of the AIC2A (mIL-3 receptor) mRNA in mouse (16), the failure to identify the hIL-3 receptor cDNA may be due to its low abundance.

Another possibility is that, unlike mice, humans do not have two homologous genes. In this case, the IL-3 binding protein, like IL-3, may have only weak conservation between human and mouse. Alternatively, the KH97 protein may be a component of both the hIL-3 and hGM-CSF receptors. IL-3 and GM-CSF induce tyrosine phosphorylation of similar sets of proteins (5) and they have overlapping biological activities (1-3). In addition, binding of hGM-CSF to its receptor is partially blocked by hIL-3 and vice versa (9-11), although IL-3 and GM-CSF have no structural homology. The KH97 protein may be shared between the hIL-3 receptor and the hGM-CSF receptor—i.e., the KH97 protein forms the high-affinity receptor for hGM-CSF with the α chain of the hGM-CSF receptor and it also forms the high-affinity receptor for hIL-3 with an unidentified protein which may or may not bind hIL-3 by itself. If this is the case, the cross-competition as well as overlapping biological activities of the two factors may be explained. It is of interest that no cross-competition of binding between mIL-3 and mGM-CSF has yet been reported. If the AIC2A protein forms the high-affinity mIL-3 receptor with an unidentified protein and the AIC2B protein is the β chain of the mGM-CSF receptor, then there may be no cross-competition between mouse factors. As the mouse low-affinity receptor for GM-CSF has not yet been isolated, at present we are not able to test this hypothesis by using cotransfection with either the AIC2A or the AIC2B cDNA. We have examined the possibility that the low-affinity hGM-CSF receptor may form a high-affinity receptor with either the mouse AIC2A or the AIC2B protein; however, none of these combinations resulted in high-affinity binding with either mouse or human GM-CSF. If there is another human AIC2 homologue which binds hIL-3 in a manner analogous to mouse AIC2A, cross-competition between hIL-3 and hGM-CSF may occur due to another shared component. If this component is present abundantly in mouse, cross-competition may not be observed. In any case, it is important to find whether humans have two AIC2 homologues and also whether mouse AIC2B is the β chain of the mGM-CSF receptor.

Neither the α nor the β chain of the GM-CSF receptor has a tyrosine kinase consensus sequence, and GM-CSF did not induce tyrosine phosphorylation in the NIH 3T3 transfectants expressing the α and β subunits of the GM-CSF receptor (T.K., unpublished data), yet GM-CSF induces tyrosine phosphorylation in hemopoietic cells (5). Thus, signal transduction through the GM-CSF receptor must require additional component(s). To understand the molecular mechanisms of signal transduction it is of particular importance to identify those additional component(s) required for signal transduction.

During the preparation of this manuscript, Metcalf *et al.* (26) reported that transfection of the α subunit of hGM-CSF receptor cDNA in a mGM-CSF-dependent mouse cell line resulted in only low-affinity binding for hGM-CSF and a high

concentration of hGM-CSF stimulated proliferation. Inability of the human α subunit to form a high-affinity receptor in mouse cells is consistent with our result that cotransfection of the human α subunit cDNA and the mouse AIC2A or AIC2B cDNA did not result in a high-affinity binding for hGM-CSF in COS-7 cells. However, the mechanism by which the low-affinity hGM-CSF receptor transmits a growth signal remains unclear.

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REVIEW ARTICLE

The Structural and Functional Basis of Cytokine Receptor Activation: Lessons From the Common β Subunit of the Granulocyte-Macrophage Colony-Stimulating Factor, Interleukin-3 (IL-3), and IL-5 Receptors

By Christopher J. Bagley, Joanna M. Woodcock, Frank C. Stomski, and Angel F. Lopez

CYTOKINE RECEPTORS are cell-surface glycoproteins that bind specifically to cytokines and transduce their signals. These receptors enable cells to communicate with the extracellular environment by responding to signals generated in the vicinity or in other parts of the organism. Thus, the initial binding of cytokines to their receptors is a key event that occurs rapidly, at very low cytokine concentrations, is usually virtually irreversible, and leads to intracellular changes resulting in a biologic response. The biologic response can vary between cytokine receptors and from cell to cell but in general it involves gene expression, changes in the cell cycle, and release of mediators such as cytokines themselves.

Cytokine receptors function as oligomeric complexes consisting of typically two to four receptor chains that may be the same or different. In single subunit receptors the subunits fulfill the dual role of binding to cytokines and signaling. Examples of receptors that use a single type of subunit are those for growth hormone (GH), erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), and thrombopoietin (TPO). In multi-subunit receptors the different subunits may perform specialized functions such as ligand-binding or signal transduction. Multi-subunit receptors may consist of two subunit types such as the receptors for granulocyte-macrophage CSF (GM-CSF), interleukin-3 (IL-3), and IL-5 where an α subunit is specific for each ligand and a β subunit is common to all three (β_c), with both chains participating in signaling. The IL-6 receptor also contains two subunit types, IL-6R α and gp130. However, in this case the function of each chain is more exclusive, with IL-6R α being the major binding protein with no direct role in signaling, and gp130 being the signal transducer. Receptors that contain three different subunits are the CNTF receptor (CNTFR), formed by the CNTFR α chain, gp130, and the leukemia inhibitory factor (LIF) receptor, and the IL-2 receptor (IL-2R) which consists of the IL-2R α chain or α_c (which is not a typical member of the cytokine receptor family), IL-2R β , and IL-2R γ , with the latter two being the signaling molecules.

The cloning of cytokine receptors has shown a striking structural and functional conservation which has justified their distinct grouping into the cytokine receptor superfamily.

However, it is becoming clear that within this superfamily, structurally similar subfamilies exist whereby some receptors or receptor subunits are more related to each other than to other members of the receptor superfamily. For example the recently cloned receptor for TPO (TPOR) is more closely related to the EPO receptor (EPOR) and β_c than to other cytokine receptors.

In functional terms some receptors have subunits that subserve similar functions. For example, the common β subunit (β_c) shared by the GM-CSF, IL-3, and IL-5 receptors is functionally analogous to gp130, which is the common subunit of the IL-6, CNTF, cardiotrophin, oncostatin M, LIF, and IL-11 receptors, to IL-2R γ which is shared by the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15, and to the common subunit of the IL-4 and IL-13 receptors.^{1,2} These common subunits have the dual function of affinity-converting the initial cytokine binding into a high-affinity state, and of being the major signal transducer in each of these receptor systems. The communal nature of these subunits helps explain much of the overlapping activities of the different cytokines in each receptor system.

This review focuses mostly on these communal subunits and, in particular, on the structure of the common β subunit (β_c) of the human GM-CSF, IL-3, and IL-5 receptors and the mechanism of activation of this receptor family. This review does not address the activation pathways and signaling molecules associated with β_c following receptor activation.

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Table 1. Sequences of the CRM From Various Signaling Subunits of Cytokine Receptors Are Compared

CRM:	β_c1	β_c2	LIFR1	LIFR2	gp130	TPOR1	TPOR2	EPOR	IL2R β	GHR	PRLR
β_c1		18.0	12.0	17.0	17.7	14.9	16.1	15.7	17.6	14.7	16.2
β_c2	23.2		13.7	18.4	20.8	19.8	19.1	23.1	16.3	15.3	14.0
LIFR1	14.7	16.2		20.3	17.7	14.0	15.6	18.4	12.4	12.6	21.6
LIFR2	15.4	16.6	19.6		29.5	11.7	15.0	18.8	16.3	13.6	18.4
gp130	16.8	22.7	18.6	21.3		15.4	17.7	17.4	14.7	17.9	24.9
TPOR1	16.6	16.8	14.9	12.8	16.4		19.5	28.2	14.9	18.6	21.1
TPOR2	15.7	17.5	17.2	16.9	18.8	17.2		21.3	17.4	15.5	19.7
EPOR	15.6	20.4	15.7	18.6	19.4	26.6	20.7		18.5	17.9	19.6
IL2R β	17.4	20.5	11.9	16.3	14.4	17.7	15.5	16.8		17.2	16.9
GHR	15.7	18.5	13.7	15.6	21.7	21.3	17.5	18.1	14.4		32.0
PRLR	19.1	18.6	19.8	20.3	27.5	23.2	17.6	20.3	17.0	31.5	

In the top/right panel, percentage identity of aligned sequences using the Pileup and Distances modules of the University of Wisconsin Genetics Computer Group software⁸⁶ and in the lower/left panel, the percentage identity determined using ClustalW.⁸⁷

tion, topics covered by recent reviews elsewhere.³⁻⁵ This review discusses recent modeling, mutagenesis, and functional studies on β_c likely to provide a paradigm on which predictions on other communal subunits may be based. In particular, the identification of regions important for the binding of GM-CSF, IL-3, and IL-5 may offer a novel strategy to interfere with the function of several cytokines at once at the cell surface. Evidence is also reviewed and presented that subtle differences in the way the communal subunits associate with the major binding subunits may exist, which has implications for the general mechanism of receptor activation and the biologic function of cytokines.

GENE ORGANIZATION OF β_c AND OTHER CYTOKINE RECEPTOR SUBUNITS

Molecular cloning of the cDNA encoding human β_c ⁶ predicts the protein to be an 880-amino acid molecule with four 100-amino acid extracellular domains related to fibronectin type III domains, a single membrane-spanning sequence and some 450 intracellular residues. The four extracellular domains are comprised of seven β strands (denoted A-G) and are organized in two cytokine receptor modules (CRM). Subsequent analysis of the gene structure of β_c and that of related receptors, and comparison with protein structures based on the paradigm of the GH receptor (GHR) suggest a relationship between gene organization and functional regions in the receptor.

Conservation of both position and phase of intron/exon boundaries has been noted in cytokine receptor genes.⁷ The organization of the genes encoding the mouse β chains, the communal β chain AIC2B, and the IL-3-specific β chain AIC2A have been determined.⁸ As with other genes from the cytokine receptor superfamily, each 100 amino acid fibronectin type III domain is encoded by two exons. The intron/exon boundaries that delineate the ends of each domain are of the phase 1 type in which the intron disrupts the codon after the first nucleotide. The intervening intron/exon boundaries for domains one and three of AIC2A and AIC2B are of type 2 where the intron disrupts the codon after the second nucleotide, and type 0 for domains two and four with the intron interrupting the reading frame between codons. This pattern of intron/exon boundaries, conserved both in position and the phase of the introns, has been described as the 1-2-1-0-1 rule.⁷

As with other cytokine receptor genes, the transmembrane region and immediate cytoplasmic portion in the genes for AIC2A and AIC2B are encoded in two small exons. This is followed by a nonconserved cytoplasmic exon and a large exon encoding the C-terminus.

The gene organization of β_c differs from that of the prototypic GHR in two respects. Firstly, there is a direct duplication of the CRM, similar to that seen in the TPOR. Secondly, the region encoding the C-terminus is interrupted by an additional phase 2 intron, a feature also seen in the IL-4R,⁹ although the cytoplasmic regions of these proteins do not exhibit any especial similarity. The two CRM of β_c apparently arose as a result of a duplication because they are somewhat more closely related to each other than to other cytokine receptors (Table 1). This is similar to the leptin receptor (OBR), in which the two CRM are more similar to each other than to other receptors, but differs from other receptors such as the TPOR in which the N-terminal CRM is more closely related to the EPOR than to the C-terminal CRM, and the LIFR in which the second CRM is more closely related to gp130 than to the first CRM.

The conservation of intron/exon boundaries in the cytokine receptor family suggests that they may delineate structural or functional regions in the proteins. This is clearly the case for the boundaries between extracellular domains of the GHR, where the intron separates sequences encoding the two pairs of cysteine residues that form intramolecular disulfides and lie immediately C-terminal to the C strand. However, in GHR, the intron in the second domain interrupts the D strand. Sequence alignment¹⁰ and molecular modeling¹¹ suggest that the introns of β_c also occur between the C and D strands in the first and third domains and interrupt the D strands in the second and fourth domains. The conserved cytoplasmic exon has been found to encode sequences essential for signal transduction by β_c ,¹² and similar regions have been identified in GM-CSF receptor α chain (GMR α),¹² IL-3 receptor α chain (IL-3R α),¹³ the IL-5 receptor α chain (IL-5R α),¹⁴ and IL-2R β chain (IL-2R β).¹⁵

PROTEIN STRUCTURAL FEATURES OF CYTOKINE RECEPTORS

The sequences of the hematopoietic cytokine receptors exhibit a conserved region of approximately 200 amino acid

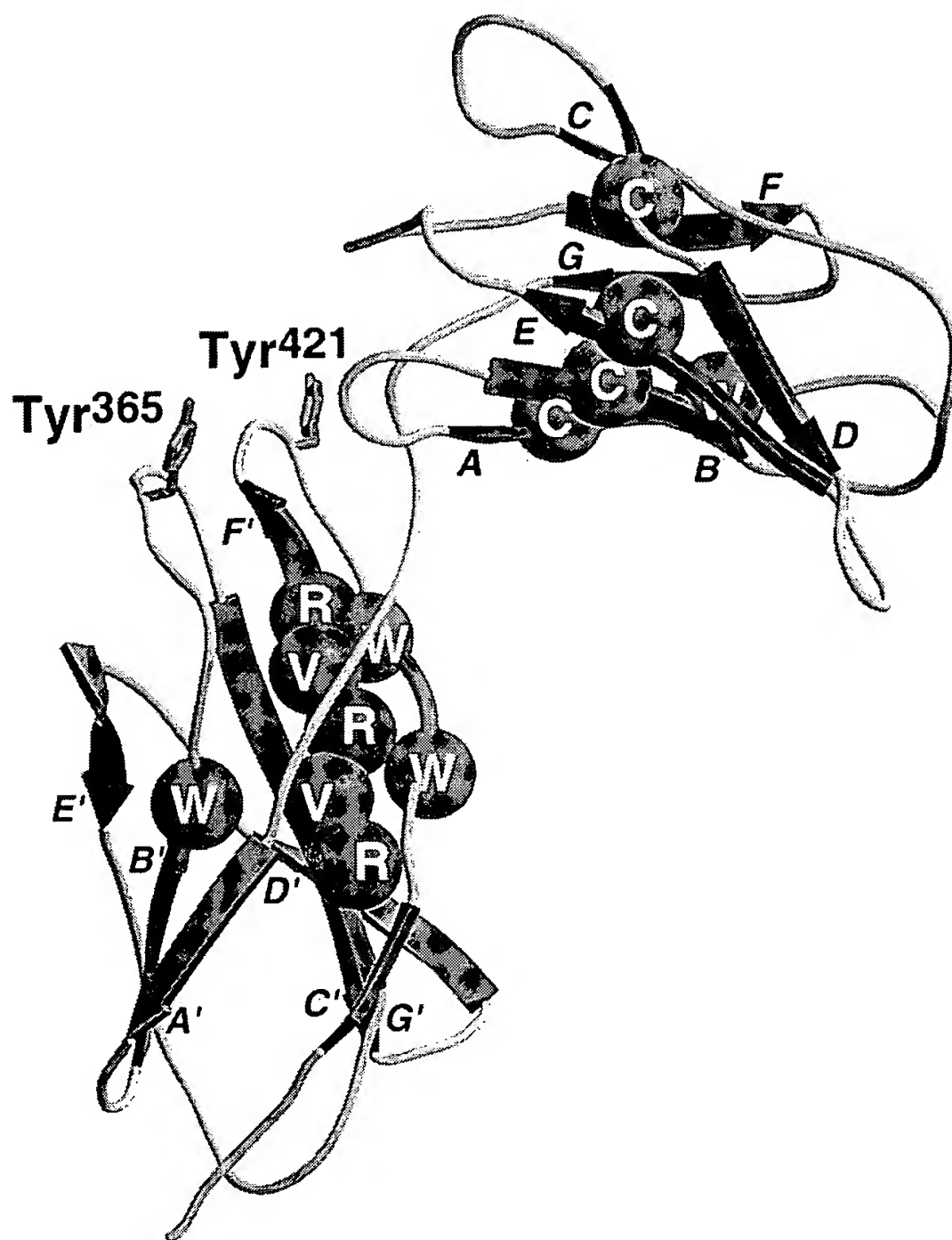


Fig 1. Ribbon diagram of the third and fourth domains of β_c with conserved residues shown by CPK spheres. Conserved residues are colored as follows: Cys, yellow; buried hydrophobics, green; Arg, blue; Trp of WSXWS sequence, purple (see cover figure). The two Tyr residues involved in ligand-binding are drawn in red stick form (see cover figure). The strands are labeled in close proximity to the arrow illustrating the direction of the relevant strand.

residues (cytokine receptor module, CRM) that was proposed to consist of two β barrel structural domains.¹⁶ Several sequence motifs may be recognized as typical of this family of receptors. Each domain contains a conserved Trp near its *N*-terminus, the first domain contains four conserved cysteine residues thought to be involved in disulfide bonds, while the second domain has two or three Pro residues near its *N*-terminus, an alternating pattern of hydrophobic residues (YXVXVRVR consensus) and an especially well-conserved WSXWS motif near its *C*-terminus. The elucidation of the structure of the GHR complexed with GH¹⁷ has provided some explanation of the role of these conserved residues. Each domain of the GHR consists of two β -pleated sheets containing the A, B, and E strands and the D, C, F, and G strands, respectively. The general topology of these strands can be seen in the β_c model (Fig 1). The conserved *N*-terminal Trp residues lie in the central, B, strand of the first sheet and constitute part of the hydrophobic cores of the

proteins. These residues interact with conserved hydrophobic residues from the central C and F strands of the opposing sheets including the YXVXVRVR motif in the second domain. The WSXWS motif exists as a β bulge facilitating interdigitation of the two Trp residues between the surface-orientated Arg side-chains of the YXVXVRVR motif in the F strand. Although the GHR has Tyr and Phe residues in place of the more usual Trp in the WSXWS motif, the aromatic rings of these residues superimpose on the Trp heterocycles in the structure of the closely related prolactin receptor.¹⁸ The elucidation of the structure of the GH/GHR complex has provided a structural paradigm on which our understanding of the structure and function of other cytokine receptors can be built.

Within the CRMs, each structural domain is related to the archetypal fibronectin type III domain but they are not closely related to each other. Although the amino acid sequences are highly divergent, this common cytokine receptor

structural motif has probably been conserved throughout mammalian evolution and has been found in other vertebrates such as birds.¹⁹ This conservation is illustrated by, firstly, the presence of sequence motifs such as the WSXWS motifs, disulfide pairs, and several other residues (eg, Trp in B strand of both domains). Secondly, in the vast majority of cytokine receptors, the cytokine receptor domains are present as pairs constituting a CRM. In addition to CRM, several receptors have recruited additional domains into their extracellular regions such as classical type III fibronectin domains, Ig domains, or cytokine-receptor second domains (IL-3, IL-5, GM-CSF receptor α chains). Although the intracellular portions of the cytokine receptors are highly variable, a membrane-proximal region of approximately 50 amino acids typically contains proline motifs and is encoded by an exon of conserved structure. This region has been implicated in signal transduction via association with members of the JAK family of protein tyrosine kinases.^{20,21}

PROTEIN STRUCTURE OF β_c

Extracellular regions. The extracellular domains of β_c bear sufficient resemblance to those of GHR to permit molecular modeling by homology. Lyne et al modeled the membrane-proximal CRM of human β_c permitting the prediction of contact sites for the ligands and receptor α -chains. The model supported the notion^{22,23} that the B'-C' and F'-G' loops in the fourth domain of β_c are involved in ligand recognition and also proposed that, like the GH receptor, the E-F loop of the third domain would be involved in ligand-binding. The major role of Tyr residues in the domain 4 B'-C' and F'-G' loops that have been identified by mutagenesis (vide infra) was not highlighted in the models of the IL-3 and GM-CSF receptor complexes. Of interest with respect to receptor activation process is the observation of the formation of disulfide-linked IL-3R α/β receptor heterodimers on binding of IL-3 leading to functional activation of these β -chains.²⁴ The N-terminal domain of β_c has seven cysteine residues and the second domain has one. The first two cysteine residues are encoded by one exon and probably form a conserved disulfide between the A and B strands. A second disulfide between the D and E strands is also predicted to occur leaving three nonconserved cysteine residues, two of which (Cys⁸⁶ and Cys⁹¹) lie in the C-D loop where they may either form an additional disulfide or be available for intermolecular disulfide formation. The remaining cysteine residues (100 and 234) are not present in the mouse β chains.

Cytoplasmic regions. Nothing is known of the tertiary structure of the cytoplasmic domains of members of the cytokine receptor family. However, in addition to their extracellular homology, several signaling subunits of cytokine receptor systems exhibit additional sequence similarities in the membrane-proximal cytoplasmic regions denoted "box 1" and "box 2"²⁵ (Fig 2). The region described as box 1 contains a basic motif (LRR), a Trp residue followed by proline-containing sequences (IPNP). In β_c confirmation of the importance of box 1 came from deletion analysis which defined a region comprising residues 456 to 487, encompassing box 1, as being critical for mediating growth response when transfected into Ba/F3 cells.¹² Sequences corre-

sponding to box 1 are found in a number of cytokine receptors where they probably serve a common function in the recruitment of kinases of the JAK family. In addition to box 1 residues, a region of β_c comprising residues 517 to 542, that includes box 2 sequences, was found to be required for the full sensitivity of the biologic response.¹² Sequences corresponding to box 2 exhibit a weak consensus of PXXLE followed by several charged residues and are encoded near the 5' of the final large exon of the signaling subunits of receptors for which the gene structures are known at a distance of typically 16 to 24 residues C-terminal to a conserved Trp residue (Fig 2). In β_c , this distance is apparently greater (39 residues), probably due to the interruption of the final exon by the third cytoplasmic intron.

These regions have been noted to be involved in the function of related receptors as well. For example, in the β chain of the IL-2R, a 13-amino acid deletion of the box 1 region resulted in a 50-fold decrease in the ability to signal in a transient proliferation assay.¹⁵ Furthermore, box 2 in IL-2R β is essential for the spectrum of activities stimulated by IL-2.¹⁵ Determination of the functional relationship between boxes 1 and 2 requires determination of the structure of this immediate cytoplasmic region.

The cytoplasmic region of β_c also contains several tyrosines that are phosphorylated after cytokine binding. A major one is Tyr⁷⁵⁰, substitution of which abolishes phosphorylation of β_c ,²⁶ which is associated with impaired viability. Tyr⁵⁷⁷, on the other hand, is essential for Shc phosphorylation.^{27,28} The role of phosphorylated receptor tyrosines in recruiting associated proteins and linking receptors to cellular functions is discussed elsewhere,⁴ but a picture is emerging whereby tyrosine-based motifs can be identified in signaling receptor subunits that couple the receptor to specific substrates.²⁹

STRUCTURAL REQUIREMENTS FOR β_c FUNCTION

Regions in β_c involved in ligand binding. As described above, the extracellular portion of β_c is comprised of two CRM. Most other receptor subunits of the cytokine receptor superfamily including the α chains for GM-CSF, IL-3, and IL-5 contain only a single copy of the cytokine receptor module. The role of the individual cytokine receptor domains of β_c is poorly understood. Studies to identify ligand binding determinants have focussed on the membrane proximal CRM, which is thought to be better positioned for interactions with ligand and α chain.

The β_c is thought to be involved in direct interaction with each of its ligands in the high-affinity receptor complex. Evidence for this comes from structure-function studies on the ligands themselves. Substitution of a conserved glutamate in the first α helix of GM-CSF,³⁰⁻³² IL-3,³³ or IL-5³⁴ abolishes high-affinity binding without affecting the ability of the mutated cytokines to bind to their specific α chains. Despite the complete loss of high-affinity binding by all the glutamate-substituted mutants, the net effect on biologic activity is not all or none, but dependent on the amino acid used to substitute the conserved glutamate. The most clear example is GM-CSF where substitution of Glu²¹ for different amino acids leads to analogues of varying potency, and in

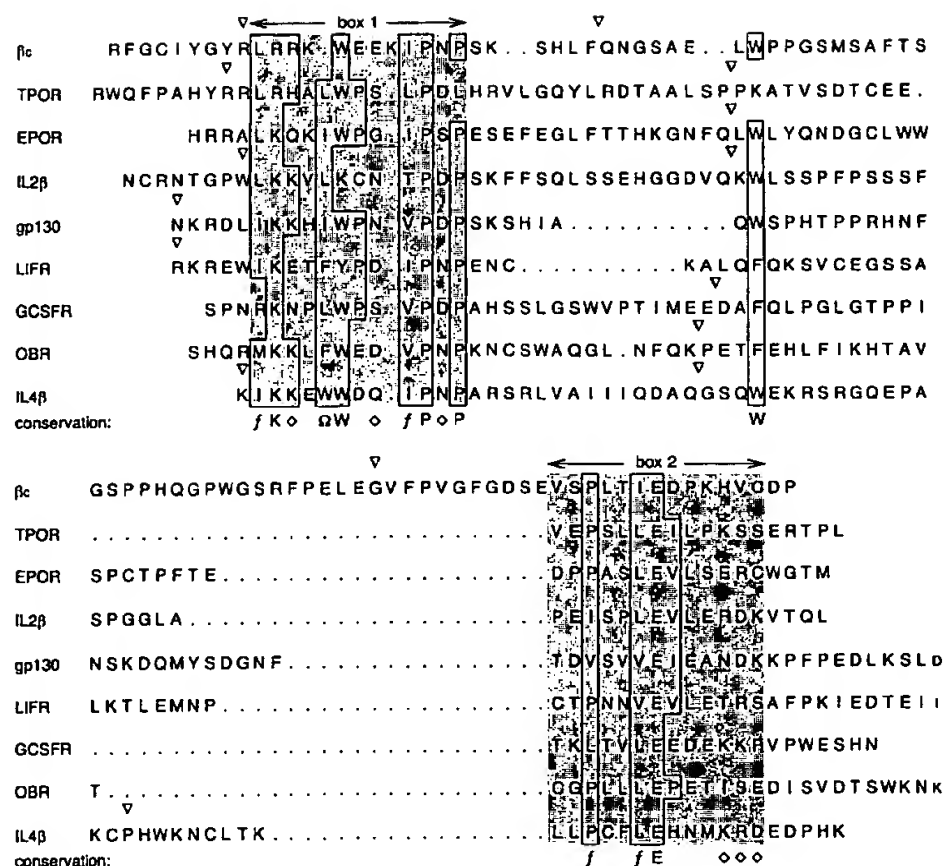


Fig 2. Sequences of the immediate cytoplasmic regions of various human receptor signaling subunits. The positions of intron/exon boundaries are indicated by (∇). In some cases, these have been inferred from the mouse genes. Conserved residues are boxed with a consensus indicated below; aliphatic (f), hydrophobic (Ω), hydrophilic (\diamond), or the conserved residue.

the case of a charge reversal (E21R, E21K), to analogues devoid of classical GM-CSF activity that behave as antagonists.^{31,35} This spectrum of biologic activities by "low affinity only binding" GM-CSF mutants suggests that residual and weak interactions with β_c , not detectable by binding experiments, are taking place. Alternatively, the Glu²¹ mutants may conformationally affect receptor dimerization and the formation of high-order GM-CSF receptor complexes required for receptor activation (see below). As β_c acts as an affinity converter in the receptor complex these findings suggest that the conserved glutamate motif is involved in β_c interaction. This is supported by cross-linking studies showing that the ligand is capable of being cross-linked to β_c , indicating that ligand and β_c are in close proximity and suggesting that the interaction between the conserved glutamate in the α helix of GM-CSF, IL-3 or IL-5, and β_c may be a direct one.

From the crystal structure of the GH-GHR complex, three regions of GHR were found to be solvent inaccessible in the ligand bound receptor complex and, therefore, represent contact sites between the ligand and the receptor.¹⁷ These regions lie in the surface exposed intervening regions between the β strands of the GHR CRM; in the E-F, B'-C' and F'-G' loops. Mutagenesis in these regions has shown important similarities and differences within the cytokine receptor superfamily.

The E-F loop has been shown to be involved in ligand binding in several cytokine receptors. Mutation studies on GHR identified Trp¹⁰⁴ in the E-F loop as being critical for interaction with GH.³⁶ No detectable binding occurred when this residue was substituted with alanine, whereas a more conservative substitution with phenylalanine reduced affinity for GH 110-fold. In the IL-5R α , Arg¹⁸⁸ in the putative E-F

loop was shown to be involved in IL-5 binding.³⁷ Alanine substitution of this residue resulted in more than a 100-fold loss in affinity for IL-5. Also, recently a residue in the E-F loop of EPOR, Phe⁹⁷, was identified by mutagenesis studies as being important for EPO binding.³⁸ Substitution of Phe⁹⁷ to alanine resulted in a 1,000-fold reduction in EPO binding affinity whereas substitution with tyrosine or tryptophan had a much less dramatic effect, suggesting that an aromatic hydrophobic residue is required for high-affinity EPO binding.

In contrast, no effect on high-affinity GM-CSF or IL-3 binding was observed by penta-alanine substitution of the PVPDP sequence predicted to lie in the E-F loop of β_c (unpublished observations, 1996), suggesting that this region is not important for ligand binding. However, the primary sequence similarity of β_c with GHR in this region is extremely weak, raising the possibility that the region targeted for mutagenesis was not analogous to the E-F loop of GHR. Further studies in β_c may yet reveal a role for this region in ligand interaction.

A second region in GHR, the B'-C' loop, was identified as being involved in interaction with GH.³⁹ Mutagenesis studies identified Trp¹⁶⁹ in the B'-C' loop as being a key hydrophobic residue required for ligand binding. Previously, the predicted B'-C' loops of IL-6R α ,⁴⁰ IL-2R β ,⁴¹ and AIC2A⁴² were implicated as being involved in ligand binding. Intriguingly, as with GHR, the residues identified in each case include a large aromatic hydrophobic residue. This suggests, as has been found with Trp¹⁰⁴ and Trp¹⁶⁹ in the GH-GHR complex, that hydrophobic interactions are important for ligand binding in many cytokine receptors.

In β_c , the putative B'-C' loop was targeted for mutagenesis in two independent studies.^{22,43} In both studies His³⁶⁷

was identified as being important for high-affinity GM-CSF binding. Our study also showed that not only His³⁶⁷ but also the hydrophobic residues Tyr³⁶⁵ and Ile³⁶⁸ were important for IL-5 as well as GM-CSF high-affinity binding, but individually had only a minor role in IL-3 high-affinity binding.²² These findings demonstrated that GM-CSF, IL-3, and IL-5 may interact with β_c in different ways and suggested that it may be possible to develop antagonists against β_c that are selective in their effect on different ligands. This observation is in parallel to results obtained with mutant ligands, where charge reversal substitution of the conserved glutamate residue in the first α helix of GM-CSF (Glu²¹) and IL-5 (Glu¹³) facing β_c ⁴⁴ not only completely abolished the biologic activity of the cytokines but also rendered them functional antagonists. Similarly, mutation of IL-3 at Glu²² severely disrupted the biologic activity of the cytokine but, in this case, the analogues still functioned as agonists, suggesting that residual functional interactions were occurring with β_c .³³

In the IL-6R α , residues in the F'-G' loop were identified as being important for ligand binding and an aromatic hydrophobic residue, Phe²⁹⁸ was implicated.⁴⁰ In the crystal complex of GH-GHR, residues of the F'-G' loop were judged to be solvent inaccessible, suggesting an interaction between this loop of the receptor and the ligand.¹⁷ However, mutagenesis studies showed that there was no productive interaction between GH and this loop in the receptor.³⁹ This is in direct contrast to the role of the F'-G' loop in β_c in which a single residue, Tyr⁴²¹, has been identified as playing a key role in high-affinity binding of all three ligands.²³ Indeed, functional studies showed a loss in IL-3-stimulated activation of STAT5 greater than 10,000-fold. Significantly, a mutant β_c in which the entire putative F'-G' loop with the exception of Tyr⁴²¹ was substituted with alanine was able to support high-affinity GM-CSF and IL-3 binding, indicating that this residue alone in the context of the putative loop region was sufficient for binding.²³ Alignment of the predicted F'-G' loops of other members of the cytokine receptor family reveals the presence of hydrophobic aromatic residues in several cytokine receptors.²³ This suggests that this region may also be involved in ligand binding in other receptor systems and may represent an ideal target for small molecule antagonists. In particular, in the case of β_c , it may be envisaged that compounds targeting Tyr⁴²¹ could simultaneously inhibit the actions of GM-CSF, IL-3, and IL-5. These molecules may have therapeutic benefit in diseases such as asthma where all three cytokines have been implicated. Further support for the global importance of this region in β_c and in other receptors²³ stems from the observation that a small EPO mimetic peptide competitively binds to this region in the EPO receptor.^{45,46}

The involvement of hydrophobic residues in ligand binding sites has become a recurrent theme in mutagenesis studies to date in members of the cytokine receptor family. In GHR, 11 residues make up the functional binding epitope and form a hydrophobic core in which Trp¹⁰⁴ and Trp¹⁶⁹ form the major interaction with GH that accounts for more than 75% of the binding free energy.³⁹ Also, the residues involved in ligand interaction in GHR lie in the intervening loop regions between β strands that are flexible and in close prox-

imity to the ligand. Thus, the conservation of structural features in the cytokine receptor family maintains the structural organization of the receptor subunits so that ligand binding sites in the loops are effectively presented for efficient ligand interaction.

In terms of communal receptor subunits that interact with multiple ligands, the β_c may provide a paradigm for ligand interaction. It is clear that in β_c there are ligand-specific and shared interaction sites for GM-CSF, IL-3, and IL-5. It would be interesting to examine whether similar observations can be made with gp130, LIFR β , and the IL-2R β chain.

Regions important for structural integrity. Several studies have examined the effect of mutating the conserved structural determinants of cytokine receptors on ligand binding. Paired cysteine residues are a conserved feature in the membrane distal domain of cytokine receptors. In studies on IL-6R,⁴⁰ the prolactin receptor,⁴⁷ and GMR α chain,⁴⁸ it has been shown that mutation of these cysteines disrupted ligand binding, suggesting that these cysteine residues define the secondary structure of the receptor. In addition, these cysteines may also be involved in disulfide interactions between β_c and the IL-3R α chain.²⁴ It is anticipated that mutagenesis of cysteine residues in the membrane distal CRM of β_c will show those residues involved in this intermolecular interaction and also determine those involved in intramolecular interactions.

A tryptophan in the B' strand is a conserved feature of the cytokine receptor superfamily. In the GHR crystal structure this residue forms part of the hydrophobic core of the membrane proximal domain.¹⁷ Substitution of this residue (Trp³⁵⁸) in β_c completely abrogates high-affinity GM-CSF binding and IL-3-induced receptor activation,⁴⁹ indicating that maintenance of the hydrophobic core is crucial for receptor integrity and consequently ligand interaction.

The role of the highly conserved WSXWS motif in cytokine receptors has long been the subject of controversy. Studies have focused on this motif in several different cytokine receptors including IL-2R β ,⁵⁰ EPOR,^{51,52} IL-6R,⁴⁰ GHR,⁵³ the GMR α chain,^{48,54} and IL-3R α chain.¹³ Mutations in this motif were found to have various effects on cell-surface expression of these receptors, ligand binding, and receptor internalization, although the structural basis for these effects was not understood. However, a recent systematic study of mutants in EPO receptor has more clearly defined the structural role of the WSXWS motif.⁵⁵ One hundred WSXWS mutants representing all the single amino acid substitutions for each of the five residues of the motif were analyzed for their effect on cell-surface expression, ligand binding, and functional response. Only conservative amino acid substitutions of the tryptophan and serine residues were tolerated, although the effect of mutation was on the secretory pathway, reducing the ability of the receptor polypeptide to exit the endoplasmic reticulum and, consequently, cell-surface expression was affected. However, the affinity and functional activity of the mutant EPO receptors that reached the cell surface was unchanged, indicating that the ligand binding site was unaltered by these mutations.

From the crystal structure of the growth hormone receptor,

the WSXWS motif forms a close interaction with another conserved motif, VRXR. The two motifs bind the F'-G' loop and interact to form a hydrophilic-aromatic stack, which thereby constrains the F'-G' loop. One of the inconsistencies with WSXWS mutations in different receptors has been their effect on ligand binding affinity. This may be as a consequence of the disruption of the F'-G' loop, which makes a major contribution to ligand interaction in some receptors, as in the β_c , but not in other receptors, as in GHR. To date no WSXWS studies on β_c have been published, but on the basis of the involvement of the F'-G' loop in ligand binding we would predict that should WSXWS mutants of β_c be cell-surface expressed, there would be a dramatic effect on ligand binding.

MECHANISM OF CYTOKINE RECEPTOR ACTIVATION

Once cytokines bind to surface receptors they induce receptor clustering or oligomerization followed by receptor activation and the generation of intracellular signals. Ligand-dependent receptor dimerization or oligomerization appears to be a general feature of cytokine receptors,⁵⁶ which applies to members of the cytokine receptor superfamily as well as to members of the receptor tyrosine kinase family. Receptor dimerization/oligomerization can be seen in cases where the receptors are constituted by single chains as with *c-kit*,⁵⁷ the GH receptor,⁵⁸ EPOR,⁵⁹ and G-CSFR,⁶⁰ as well as in multi-subunit receptors such as the IL-6⁶¹ and IL-3²⁴ receptors. Receptor dimerization is more often than not induced by ligand. In the cytokine receptor superfamily, GH has been shown to induce GHR dimerization,⁵⁸ and IL-6 and IL-3 also induce dimerization of their respective receptors.^{24,61} On the other hand, EPO does not seem to play a role in dimerizing the EPOR.⁵⁹ An intermediate example is the G-CSFR where G-CSF induces the conversion of a dimeric receptor into a tetrameric one.⁶⁰

In the GM-CSF, IL-3, IL-5 receptor subfamily, intriguing differences are beginning to emerge that may have implications for the role of these receptors in hematopoietic cell function. The major difference lies in the absolute requirement for IL-3 and IL-5 for dimerization of IL-3R α with β_c , and IL-5R α with β_c whereas, in contrast, at least some of the GM-CSFR probably exists as a preformed complex. In some experiments GM-CSF has been shown to facilitate the co-immunoprecipitation of GMR α with β_c ^{62,63}; however, other experiments using mutated receptors and mutated GM-CSF analogues suggest the existence of a preformed GMR α - β_c heterodimeric complex. In the case of a mutated GMR α where the second conserved cysteine was replaced, GM-CSF was unable to bind to this receptor alone, but bound with high affinity when this mutant receptor was coexpressed with β_c .³⁰ Reciprocally, a mutated GM-CSF molecule carrying an Asp¹¹² mutation showed no detectable binding to GMR α alone, yet exhibited nearly full wild-type activity in cells expressing both GMR α and β_c .³⁰ Both cases suggest that the presence of β_c in a preformed complex with GMR α compensates for losses in the GM-CSF:GMR α binding. The intrinsic interaction of GMR α and β_c appears to be weak (with GM-CSF stabilizing the GMR α : β_c complex by 1,000-fold),⁶⁴ but nevertheless of sufficient strength to be detectable

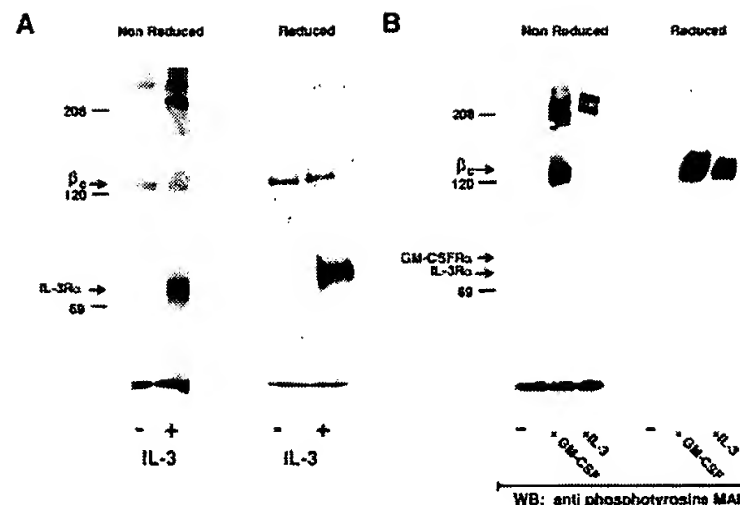


Fig 3. Disulfide-linked receptor complexes in the GM-CSF, IL-3 receptor family. (A) Immunoprecipitation of ¹²⁵I-surface-labeled primary leukemic cells with anti- β_c monoclonal antibody in the absence of ligand or in the presence of IL-3. Proteins were separated on a 7.5% SDS-PAGE gel under nonreducing or reducing (4% 2-mercaptoethanol [2-ME]) conditions. (B) Immunoprecipitation of MO7e cells with anti- β_c monoclonal antibody and immunoblotting with antiphosphotyrosine antibody. The cells were either untreated or treated with GM-CSF or IL-3. Proteins were separated on a 7.5% SDS-PAGE gel under non-reducing or reducing (4% 2-ME) conditions.

in cells coexpressing soluble GMR α and membrane-bound β_c using anti-subunit-specific monoclonal antibodies.⁶⁵ Using a similar cell-based system we have found that in stably transfected cells expressing full-length GMR α or full-length IL-3R α together with soluble β_c , antibodies to β_c detect surface staining on the former but not on the latter cells (unpublished data, 1996), supporting the notion that β_c association with GMR α and IL-3R α is fundamentally different.

It is possible that GMR α and β_c are selectively coexpressed and cotransported to the endoplasmic reticulum and the cell surface. In structural terms it is possible that E' strands and A-B loops in domain four of β_c and domain 2 in GMR α form a favorable interface allowing subunit-subunit interaction akin to the GH receptor 1 and GH receptor 2 interaction. The biologic significance of a preformed GM-CSFR complex is unclear but may facilitate a small amount of signal to trickle into the cell. It is worth noting the ubiquitous presence of the GM-CSFR in hematopoietic cells and the pivotal role it plays in functions such as survival^{66,67} and apoptosis.^{68,69} It is also interesting to note that cell survival is, of all the functions triggered by GM-CSF, the one that requires the smallest amount of GM-CSF,^{70,71} indicating that very low levels of receptor occupancy and presumably receptor dimers are required for signaling survival. It is possible that a preformed GM-CSFR complex affords a small and transient amount of "survival signaling" that gives the cell "time" to encounter the appropriate cytokine in the immediate environment.

The mechanism of receptor dimerization involves either two identical receptor subunits (homodimerization) or two different subunits of the receptor (heterodimerization). Examples of the former are the single-chain receptors. The GH, G-CSF, and EPO receptors have been shown to homodimerize followed by recruitment of the appropriate tyrosine ki-

nase and signaling. The receptor for TPO (TPOR) would also be expected to undergo homodimerization. In multisubunit receptors such as the IL-6 and IL-3 receptors, both heterodimerization and homodimerization have been shown to occur. In the case of the IL-6R, IL-6 triggers the heterodimerization of IL-6R α , the major binding subunit, with gp130, the signaling subunit.²⁵ This in turn allows the homodimerization of gp130 to a second gp130 molecule.⁷² In the case of the related CNTF receptor, CNTF heterodimerizes with gp130 while gp130 also heterodimerizes with the LIFR to initiate signaling.⁷³ In the case of the IL-3R, IL-3 triggers heterodimerization of IL-3R α with β_c .²⁴ In the GM-CSF, IL-3, IL-5 receptor family, homodimerization of β_c ^{24,63} (Fig 3) as well as heterodimerization of the GMR α and β_c (unpublished results, 1996) have been observed, both cases in the absence of stimulus. Stimulation with GM-CSF, on the other hand, leads to further heterodimerization of GMR α with β_c ⁶² in a similar fashion to IL-3-induced heterodimerization of its IL-3R α with β_c . Whether ligand induces further homodimerization of β_c is not yet clear.

Dimerization of cytokine receptor subunits has been shown to occur by covalent and noncovalent means. The dimerization of single-chain receptors has been shown to be noncovalent such as with the GHR¹⁷ but also covalent involving disulfide bonds as with the EPOR.⁷⁴ With the IL-6R and CNTFR the heterodimerization of the ligand binding, nonsignaling α subunits to gp130 is noncovalent; however, the association between the signaling subunits involves disulfide linkage.^{72,73} Dimerization of both the GM-CSF and IL-3 receptors is different to the receptors mentioned above in that a disulfide-linkage has been observed between each of the binding α subunits and β_c .²⁴ Figure 3A illustrates this point by showing that in the presence of IL-3 two high-molecular-weight complexes are induced. These have been shown to be disulfide-linked and to contain IL-3R α and β_c .²⁴ In the absence of IL-3 a 240,000 molecular-weight band is seen representing a disulfide-linked β_c homodimer.

In the IL-6, LIF, CNTF and EPO receptors, nondisulfide as well as disulfide-linked receptor dimers are associated with receptor activation as measured by phosphotyrosine reactivity of the dimers. Similarly, in the case of the GM-CSFR, both disulfide and nondisulfide-linked dimers are tyrosine phosphorylated upon the addition of GM-CSF (Fig 3). In contrast, in the case of the IL-3R, the majority of the phosphotyrosine reactivity is associated with the disulfide-linked complex and very little, if any, with the nondisulfide complexes²⁴ (Fig 3), indicating that the formation of the former is essential for receptor activation. This concept is further supported by the demonstration that prevention of this disulfide-linked association between IL-3R α and β_c by iodoacetamide inhibits receptor phosphorylation.²⁴ Interestingly, IL-3 high-affinity binding is not prevented by iodoacetamide, indicating that high-affinity binding and receptor phosphorylation are dissociable events.²⁴ These experiments and the presence of IL-3 in only the noncovalently linked α - β heterodimers²⁴ suggest a sequence of events in which IL-3 binds initially to IL-3R α and the IL-3:IL-3R α associates with β_c through CRM2, forming a high-affinity noncovalently-linked complex. The bringing together of IL-3R α

and β_c may facilitate the disulfide linkage of a free cysteine in domain 1 of β_c with a free cysteine in the *N*-terminal domain of IL-3R α . A similar interaction may occur with the unpaired Cys in the *N*-termini of GMR α and IL-5R α . The resulting effect is receptor activation by phosphorylation. A recurrent theme in receptor activation is that the major receptor signaling subunits, not only β_c but also gp130 and LIFR β in the IL-6 and CNTF receptor systems, become phosphorylated. In contrast, none of the α subunits show evidence of phosphorylation.

These experiments illustrate a fundamental difference between the IL-6R and the GM-CSF, IL-3, IL-5 receptor families in terms of the contribution of the receptor α chains. Thus, while the IL-6R α chain does not form disulfide-linked dimers with gp130 and its cytoplasmic portion is not essential for receptor activation, the GMR, IL-3R, and IL-5R α chains have been shown to require the cytoplasmic domain of the respective receptor α chains,^{12-14, 75} and at least IL-3R α and GMR α form disulfide-linked complexes (Fig 3).

It is interesting to note that in addition to GMR α or IL-3R α covalent heterodimerization with β_c , recent experiments have also noted that β_c homodimerization is sufficient for receptor activation. This has been shown using chimeric molecules expressing extracellular GMR α chain domains and cytoplasmic β_c domains. Cells expressing these chimeras together with wild-type β_c can proliferate in the presence of GM-CSF.⁷⁶ This is analogous to the constitutive activation of the EPOR by an extracellular R129C mutation which leads to receptor dimerization.⁷⁷ Similarly, chimeric EPOR/ β_c with the R129C mutation causes constitutive JAK-1/JAK-2 phosphorylation and factor-independent growth,⁷⁸ indicating that homodimerization of β_c is sufficient for signaling. Although this induced β_c homodimerization (mediated by either ligand or a mutant Cys-Cys linkage) leads to receptor activation, we have also observed the existence of spontaneous covalently linked β_c homodimers in the absence of ligand in primary cells (Fig 3A). However, these β_c dimers are not phosphorylated (Fig 3B) and phosphotyrosine reactivity is observed only when ligand is added to the cells. Similarly, β_c dimers were demonstrated by cross-linking experiments that were phosphorylated in response to ligand.⁶³ This suggests that under normal conditions the α chain is required to activate the receptor through both the initial binding of ligand and by facilitating β_c dimerization. The existence of disulfide-linked β_c homodimers in the absence of ligand, to which disulfide-linked heterodimers can be added by the presence of ligand, may favor the formation of hexameric complexes necessary for receptor activation (see below) analogous to the IL-6R system. Hexameric IL-6:IL-6R complexes have already been shown by analytical centrifugation⁷⁹ and IL-6 mutagenesis and IL-6R immunoprecipitation studies.⁸⁰ This stoichiometry is facilitated by the ability of IL-6 to interact with its receptor through three distinct binding sites, one for IL-6R α and two for gp130. The ability of gp130 to homodimerize completes and stabilizes the hexamer.

In the case of the GM-CSF, IL-3, IL-5 receptors, the stoichiometry of the ligand-receptor complex has not yet been defined. This system appears to be different from the IL-6R

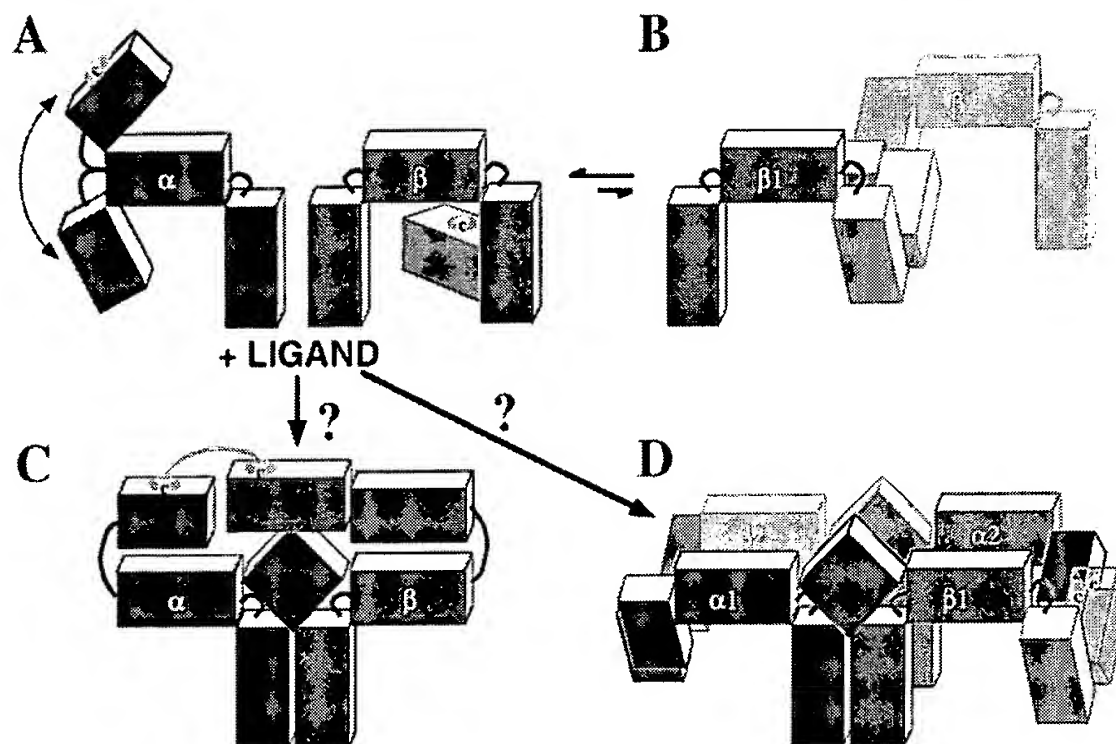


Fig 4. Schematic representation of possible GM-CSF, IL-3, and IL-5 receptor complexes. (A) The receptor α and β_c interaction in the absence of ligand. The cartoon illustrates that the N -terminus of the α chain has approximately 90° angle flexibility from the CRM. In the case of the IL-3R²⁴ and probably also of the IL-5R, the α and β_c subunits are not associated either covalently (disulfide linked) or noncovalently. In the case of the GM-CSFR, there is no disulfide linkage but there is probably a noncovalent association through domain 2 of the GMR α CRM and domain 4 of β_c . (B) In a dynamic and probably reversible manner, β_c can homodimerize by disulfide linkage. However, this is a nonproductive interaction because no tyrosine phosphorylation of β_c is seen (see also Fig 3).⁶³ (C) The binding of ligand to these receptors may then give rise to a trimer with a ligand: α : β_c stoichiometry of 1:1:1. However, the 180° angle required for the α subunit to link up with β_c makes this possibility unlikely. Instead, (D) illustrates a more likely ligand: α : β_c stoichiometry of 2:2:2 where the α subunit of receptor 1 forms a disulfide bond with β_c of receptor 2 and the α subunit of receptor 2 forms a disulfide bond with β_c of receptor 1. The free cysteines in the α and β_c subunits are illustrated.

system in that so far ligand has been found to bind its receptor through two distinct sites; one comprising helix D^{30,81} and probably also helix C⁸² that interacts with the specific receptor α chains, and a second involving mainly the conserved Glu motif in Helix A^{30-32,83} that interacts with the B'-C' and F'-G' loops of domain 4 in β_c .^{22,23,43} The formation of disulfide-linked α - β_c heterodimers is critical in this ligand receptor complex²⁴ (Fig 3). These are likely to occur through free cysteines present in the N -termini of each α subunit and a free cysteine present in domain 1 of β_c . It is worth noting that the N -termini of the GMR α , IL-3R α , and IL-5R α subunits are not classical cytokine receptor modules and are not present in other members of the cytokine receptor superfamily. Furthermore, an uneven number of cysteines are present in each of the three α subunits, highlighting the availability of at least one cysteine for intermolecular bonding. From molecular modeling of β_c the free cysteine in β_c is likely to be at position 86 or 91 in domain 1. The remaining cysteines are in conserved positions which from cytokine receptor sequence alignment and modeling are likely to be important in intramolecular bonding and maintenance of structural integrity. Figure 4 shows the α and β_c subunits of this class of cytokine receptors highlighting the putative free cysteine and the predicted approximate 80° angles which link each domain to the next. Of note is the flexibility of the linkage of the N -terminal domain of the α chain to the CRM, which would allow it to flex toward or away from the membrane

(Fig 4A); however, it would be extremely unlikely to permit an angle of more than 125° . The IL-3R α subunit and probably also the IL-5R α subunit are not associated with β_c in the absence of ligand. However, the GMR α subunit and β_c are probably pre-associated through interaction of their membrane proximal domains. In the absence of ligand β_c homodimerizes probably by disulfide linkage of its free cysteines (Fig 4B); however, this is a nonproductive interaction because no phosphorylation of β_c is seen (Fig 3).⁶³ The presence of ligand may induce complexes formed by trimers or hexamers. The formation of a trimer (Fig 4C) seems unlikely as it would require the hinge between the N -terminus and the CRM of the α subunits to exhibit an unusual flexibility to allow the α and β_c subunits to form a disulfide bridge. Thus, a trimer in which ligand contacts both receptor subunits which in turn disulfide-link to each other seems unlikely because an angle of about 180° would be required (Fig 4C). Instead, we favor the possibility that, after ligand binding, two disulfides are formed, one involving an α subunit of receptor 1 with a β_c subunit of receptor 2, and a second disulfide involving the β_c subunit of receptor 1 with the α subunit of receptor 2 (Fig 4D). This model is consistent with the possible angles between receptor domains, the existence of high-order molecular-weight complexes by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)²⁴ and the formation, under certain conditions, of ligand dimers themselves.^{84,85} Ultimately,

analytical centrifugation and crystallization of the ligand-receptor complex may resolve this issue, but it is nevertheless apparent that the GM-CSF, IL-3, IL-5 receptor system offers a new type of structural assembly and dynamics of receptor subunit interaction. It remains to be seen whether the lessons learned from this system will apply to other members of the cytokine receptor superfamily.

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